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FROM THE INTRODUCTION:

The research worker and the clinician often have neither time nor opportunity to read the literature dealing with subjects not directly related to their work, and they will be all the more apt to lose sight of the results of the latest investigations, as the relevant literature is scattered in a great number of different periodicals - often written in foreign languages. This applies above all to the field of antibiotics and chemotherapy, and it was therefore decided to meet the urgent need for collective reviews in the form of annual surveys reporting the latest results of research in this field. The world literature will be critically reviewed and referred to in detailed bibliographies. Thus the surveys will enable the specialist to keep up to date in his own field, at the same time supplying a review of the latest developments to all those not so well versed in this special field. The reports will further save the research worker the trouble of obtaining the original literature and enable all other scientists to overcome the difficulties of making themselves familiar with this particular field of medicine. With this aim in view, the annual survey will help to maintain contact between scientific research, clinic and daily practice.

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ALLOXAN DIABETES IN THE RAT: RECOVERY FOLLOWING ESTROGEN TREATMENT[1]

RICARDO R. RODRÍGUEZ[2]

Instituto de Biología y Medicina Experimental, Costa Rica 4185, Buenos Aires, Argentina

THE influence of sex glands and sexual hormones upon the incidence of diabetes in the white rat is demonstrated by the following facts: 1) after subtotal pancreatectomy (removal of about 95% of the gland), the frequency of diabetes is higher in males than in female rats (Foglia, 1945; Rodriguez, 1950 c); 2) ovariectomy increases greatly the incidence of pancreatic diabetes and castration slightly decreases it (Foglia, Schuster and Rodríguez, 1947; Foglia and Penhos, 1952); 3) the restitution of the ovaries by means of a graft leads to a considerable protection in subtotally pancreatectomized animals (Foglia, 1949); 4) estrogen administration, specially diethylstilbestrol, is followed by a biphasic effect; at the beginning there is an increase of the blood sugar level and glycosuria in partially pancreatectomized rats (Ingle, 1941; Rodríguez, 1950 b), in normal forced fed rats (Ingle, 1941), or in alloxan diabetic rats (Houssay and Mazzocco, 1946; Ingle, Nezamis and Prestrud, 1947). But this stage lasts for only about one month and is followed by the permanent protective action, with normal values of glycemia (Rodríguez, 1950 b). This protective action was obtained with estradiol benzoate (Foglia, Schuster and Rodríguez, 1947), diethylstilbestrol, estrone, dienestrol, ethynil estradiol, ethynil testosterone (Lewis, Foglia and Rodríguez, 1949-1950; Rodríguez, 1950 c), pheno-

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[1] This investigation was supported in part by a research grant from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Service. Presented at the XIX International Physiological Congress at Montreal, Canada, August 31–September 4, 1953.

[2] Fellow of the E. Santamarina Foundation.

cycline (Rodríguez, 1950 a) and monobenzyl ether of stilbestrol (Rodríguez, unpublished experiments). Conversely, androgenic substances, like testosterone and methyl testosterone, increases the incidence of diabetes in partially pancreatectomized rats (Lewis, Foglia and Rodríguez, 1949-1950). The protective action of estrogens is accompained by hypertrophy and hyperplasia of the islets of Langerhans of the pancreas, in normal rats, guinea pigs and cats (del Castillo and Sammartino, 1937; Cardeza and Rodríguez, 1949), in subtotal pancreatectomized rats (Lewis, Foglia and Rodríguez, 1949-1950; Cardeza, 1950) and in partially pancreatectomized-adrenalectomized rats (Rodríguez, 1950 c); 5) when the estrogen treatment is discontinued, the rats remain free from diabetes during six months (Rodríguez, 1950 a; Rodríguez and Martínez, 1950).

The experiments here reported show that in alloxan diabetic rats, with forced feeding, the prolonged administration of estradiol benzoate, associated or not with insulin, has a curative action.

METHODS

White rats of both sexes, from the Institute strain, were used. When they reached 150 gm. of body weight they were injected with alloxan, 40 mg./kg. body weight, intravenously. A week later, a 7 hour fasting blood sugar was performed and all the rats with more than 150 mg.% were selected for the experiment. A group of alloxan injected non diabetic rats was added and these animals are presented in the results as non diabetic controls.

The diabetic animals were divided into several groups: a) non-treated; b) treated with regular insulin, about 7 units per rat per day, in order to avoid glycosuria; c) injected subcutaneously, during six months, with estradiol benzoate, 15 μg./rat/day, mixed with 25 mg. of lactose suspended in 0.5 cc. saline solution (0.9% Na Cl); d) injected with both, insulin and estradiol, as in groups b and c.

The composition of the forced fed diet was:

Yeast	100 gm.
Dry milk	600 gm.
Salt mixture No. 2	40 gm.
Casein	120 gm.
Mazola oil	100 gm.
Cod liver oil	12 gm.
Water	1400 gm.

The final composition, in per cent, was:

Water	59.78	
Dry residue	40.22	100.00
Carbohydrate	14.77	36.72
Protein	14.37	35.73
Fat	7.37	18.32
Ash	3.71	9.22

The animals received a total daily dose of 20 cc., divided in 2 rations. They were fed from 8 to 8:45 a.m., and from 4:30 to 5:30 p.m. The mixture was warmed up to 35° C.,

immediately before feeding. In order to avoid alimentary shock, a total daily ration of 10 cc. was given at the beginning, increasing by 1 cc. daily, so that ten days later the animals were receiving the full daily dose of 20 cc.

The rats were kept in cages in groups of 8 in each. The animals receiving insulin were kept separately in a single cage, at a temperature of 22° to 23° C. They drank water at will. The urine of each animal was collected preserved under toluene and every 24 hours its glucose content was determined by Benedict's method. The body weight and blood sugar were determined monthly; blood samples in all the animals were drawn from the tail 7 hours after the morning forced feeding (in the morning of the day in which glycemia was performed, insulin injection was discontinued). Glycemia was determined by the Somogyi-Nelson method.

After 6 months of injections, treatment was discontinued and the animals with normal blood sugar were forced fed during another 3 months' period.

An animal was considered to be cured when repeated blood sugar determinations were normal (between 80 and 120 mg.%), the insulin requirements were zero (no glucose in urine) and the condition persisted three months after the estradiol treatment was discontinued.

At the end of the experiment the animals were killed, the organs weighed and the pancreas fixed in Helly solution; paraffin sections of 4 microns were made and stained with hematoxylin-eosin, Gomori's chromic hematoxylin and Heidenhein's azocarmine, following Gomori's modification for the Langerhans islets. Autopsies were carried out at frequent intervals during the experiment in order to appreciate, histologically, the effects of estradiol treatment, alone or associated with insulin, upon the islets of the pancreas.

RESULTS

Table 1 shows that none of the diabetic rats, treated or not with insulin, was cured during the six months' observation period, although insulin treatment markedly decreased the mortality.

The administration of estradiol benzoate, specially in those animals in which the treatment was associated with insulin, cured a high proportion of the diabetic rats (Table 1). This regression persisted during the three months after the treatment was discontinued.

TABLE 1. CURATIVE ACTION OF ESTRADIOL BENZOATE AND INSULIN, SEPARATED OR ASSOCIATED, ON ALLOXAN DIABETES IN RATS, AFTER SIX MONTHS OF TREATMENT AND THREE MONTHS AFTER ITS INTERRUPTION. FORCED FEEDING

	Mortality		Cured	
	No. of rats	%	No. of rats	%
Diabetic non treated	7/27	26	0/27	0
Insulin	1/29	3	0/29	0
Estradiol (15μg./day)	7*/19	36	9/19	47
Estradiol and insulin	9†/42	21	29/42	69

* Died during the initial period of exacerbation of diabetes.
† Two died during the initial period of exacerbation of diabetes.

The initial and final blood sugar values of the four groups of diabetic rats are shown in Table 2.

TABLE 2. INITIAL AND FINAL BLOOD SUGAR OF DIABETIC RATS FORCED FED BY STOMACH TUBE DURING SIX MONTHS

Glycemia (mg. per cent)		No. of rats					
		Less than 100	100 to 150	150 to 200	200 to 250	250 to 300	More than 300
Diabetics (non treated)	Initial	0	0	9	5	7	2
	Final	0	0	5	6	8	4
Insulin	Initial	0	0	4	7	11	4
	Final	0	0	9	8	5	4
Estradiol	Initial	0	0	5	3	1	3
	Final	3	6	0	0	2	1
Estradiol and insulin	Initial	0	0	8	6	8	6
	Final	8	16	0	0	1	3

The insulin requirements of alloxan diabetic rats non injected with estradiol remained unchanged during the six months' period (between 6 and 8 units of regular insulin/rat/day; average 7.3 units). In the diabetic rats treated with estradiol plus insulin, the regression of diabetes was accompanied by a decrease in the insulin requirements down to zero units, in 3 to 4 months (Fig. 1).

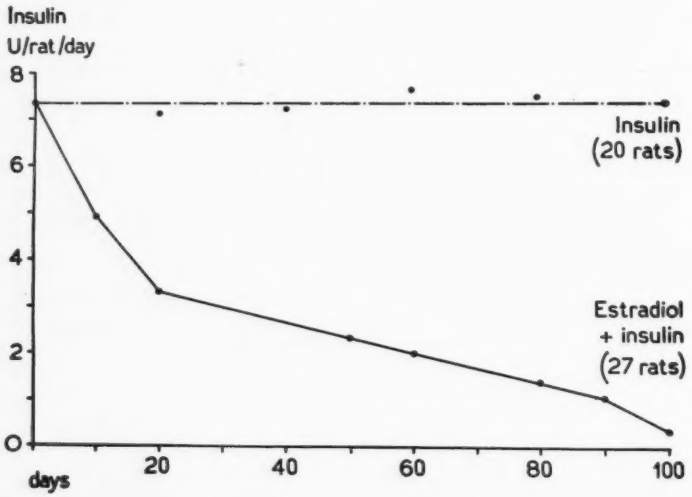

FIG. 1. Insulin requirements, in order to avoid glycosuria, of forced fed alloxan diabetic rats cured by means of estradiol benzoate.

Body weight changes in these forced fed diabetic rats shows that the non treated diabetic animals grew less, compared with the alloxan injected non diabetic ones (Fig. 2). The insulin treated rats grew like the non diabetic

controls. An almost complete inhibition of growth could be observed with the daily administration of 15 μg. of estradiol benzoate, for six months, but the action was temporary and disappeared when estradiol was discontinued.

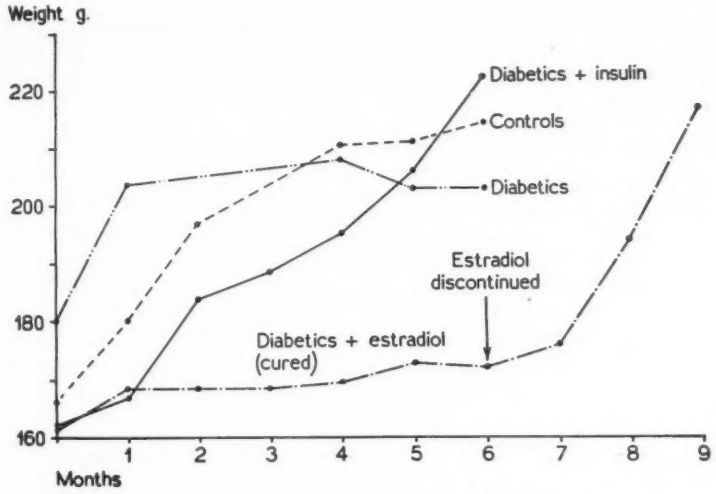

FIG. 2. Growth curve of forced fed alloxan diabetic rats.

Table 3 shows the results of the autopsies, performed at the end of the experiment.

TABLE 3. WEIGHT OF ORGANS (IN MG. PER 100 GM. BODY WEIGHT) OF FEMALE ALLOXAN DIABETIC RATS, FORCED FED DURING SIX MONTHS

	Non diabetic controls	Diabetic	Diabetic plus insulin	Diabetic (cured)*
No. of rats	7	2	6	6
Weight (gm.)	221	197	226	222
Hypophysis	5.7	5.1	4.8	12.6
Adrenals	28.5	38.3	25.9	29.2
Kidneys	996	1273	880	940
Ovaries	23.7	17.2	20.0	13.8
Uterus	200	148	151	180
Heart	332	350	334	315

* Three months after estradiol administration (15μg./day during six months) was discontinued.

Histological examination of the pancreas (performed by Dr. A. F. Cardeza): the diabetic rats treated with estradiol, alone or associated with insulin, and cured showed islets of Langerhans of normal shape and size (hematoxylin-eosin stain). The beta cells (Gomori's chromic hematoxylin), appeared normal or slightly diminished in number, with well conserved

and normally stained granules. In the animals treated with estradiol, in which diabetes was only ameliorated, the islets of Langerhans were fewer in number and reduced in size; beta cells were decreased in number and some had less granules and vacuolization of their protoplasm.

The pancreas of diabetic animals stained with the same techniques showed only a few islets of small size; beta cells were reduced in number or absent and with few granules, vacuoles in the protoplasm and pycnotic nuclei.

The insulin treated rats, with moderate or severe diabetes, showed islets reduced in size and in less number as compared with normal animals. The beta cells were also less in number, but the granules were well conserved in the majority of the islets; some beta cells showed regressive changes (Fig. 3).

DISCUSSION

These experiments show that, in the conditions under which the observations were carried out, the diabetes produced in the rat by the injection of alloxan remains unchanged in the first six months. Some rats died during this period as the results of cachexia or diabetic coma.

Insulin administration, in amounts sufficient to avoid glycosuria, have no curative action, although the beneficial effects upon diabetes are shown by the fact that: a) the growth curve and general conditions of the animals were like that of the normals (Fig. 2); b) the onset of cataracts was zero (Rodríguez and Krehl, 1951; Rodríguez, 1953); c) the mortality rate, in six months, was very low (Table 1); d) the increase in weight of the adrenals and kidneys, observed in diabetic rats, can be prevented by means of insulin (Table 3); e) there is a prevention of the changes seen in the renal function (inulin clearance), and in kidney histology (Rodríguez, Braun, Menendez and Mancini, unpublished observations).

The treatment with estradiol alone, in forced fed diabetic rats, produced, at the beginning, the well known exacerbation of diabetes. During this period, which lasted about one month, some of the animals died (7 out of 19; 36%). Those which survived became cured in a high proportion 9 out of 12; 75%), specially those animals with fasting blood sugar between 150 and 250 mg. %. Rats with values higher than 250 mg. per cent generally died during the initial period of exacerbation of the disease and the majority of the surviving ones remained diabetic in spite of the estradiol treatment.

When estradiol injections were associated with insulin, preventing the blood sugar rise, the mortality during the first period was much lower (2 out of 42); and a higher proportion of regression of diabetes was obtained. The difference between these animals and those treated with estradiol alone was specially marked in the rats with glycemia higher than 250 mg.

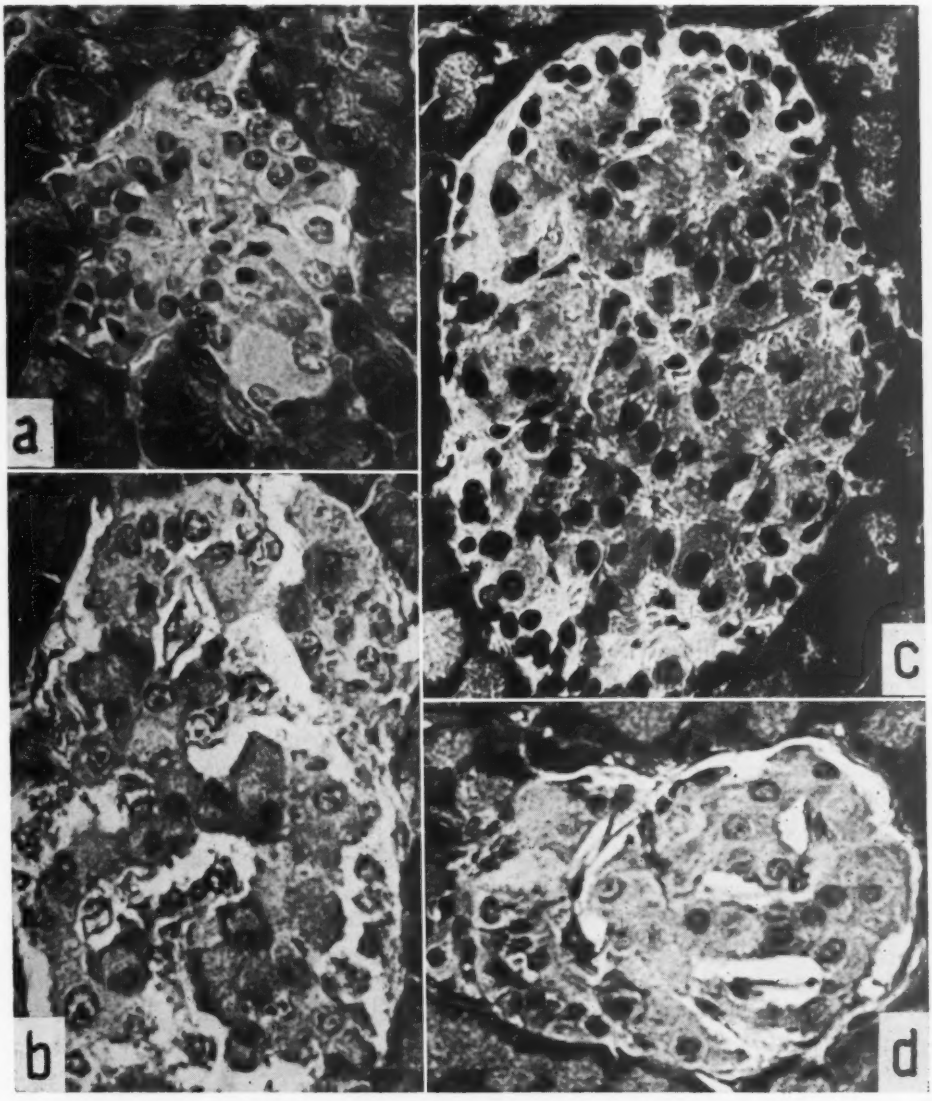

FIG. 3. *A*) Atrophic islet of Langerhans from an alloxan diabetic rat (4 months of diabetes); degranulated and vacuolized beta cells. *B* and *C*) Islets from rats cured by means of estradiol and insulin (hypertrophy in case C); beta cells well granulated. *D*) Islet from insulin treated diabetic rat; small size and beta cells slightly degranulated. Gomori's chromic hematoxylin ×250.

%, in which insulin, preventing the harmful effects of hyperglycemia upon the islets of Langerhans allowed the hypertrophy and hyperplasia of the islets of the pancreas under estradiol action and, subsequently, the regression of the diabetic state in the majority of the rats.

The fall of the insulin requirements of the cured rats, from 7.3 to 0 units per day was obtained in 3 to 4 months, in close agreement with the time required for the pancreas to show hypertrophy and hyperplasia of the islets of Langerhans.

The associated metabolic effects of estradiol treatment which resulted in a cessation of growth cannot be an important factor in the cure of the diabetic state in these animals, injected or not with insulin, because the untreated diabetic animals remained diabetic in spite of the cessation of growth observed in the last five months of the experiment. When estradiol treatment was discontinued, the growth of the cured rats was restored to normal, but these animals remained with normal blood sugar and without glycosuria.

The increase in weight of the pituitary and adrenal glands, observed during estrogen treatment, partially regressed three months after the injections were discontinued, in agreement with the results obtained in partially pancreatectomized rats injected with phenocycline (Rodríguez, 1950 a).

Estrogens can exert their protective action: a) through the hypophysis; b) through some other glands; c) on the peripheral tissues; d) directly on the pancreas. The actions a, b and c are not analyzed in this work. The hypertrophy and hyperplasia of the islets of Langerhans was previously reported in publications on the preventive action of estrogens on the onset of diabetes in partially depancreatized rats (Lewis, Foglia and Rodríguez, 1949-1950; Cardeza and Rodríguez, 1949; Cardeza, 1950). The protective action of estrogens was maintained in the majority of the rats when treatment was discontinued.

SUMMARY

White rats made diabetic after alloxan injection were forced fed a special diet and treated with estradiol, alone or associated with insulin, during six months.

The following results were obtained:

1) The non treated rats remained diabetic and some of them died during the six months' observation period.

2) The administration of insulin had no curative action, although the animals were in very good conditions and the mortality was almost zero.

3) The treatment with estradiol alone cured 47% of the rats (9 out of 19), specially those with initial hyperglycemia not exceeding 250 mg. per cent.

4) The association of both insulin and estradiol cured 69% of the rats (29 out of 42). The recovery of diabetes was accompanied by a fall of the insulin requirements from 7 to 0 units per rat per day, in 3 to 4 months of treatment.

5) The histological picture of the pancreas showed practically normal islets of Langerhans, only in the cured rats.

Acknowledgments

I wish to express my sincere appreciation to Dr. Adolfo F. Cardeza for the microphotographs and histological examination of the pancreas.

We are grateful to the Schering Corporation, Bloomfield, N. J., for the estradiol benzoate, to the Glaxo S. A., for the milk powder and to Fabrica Argentina de Fermentos for the yeast used in this study.

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THE INFLUENCE OF CERTAIN STEROIDS ON THE OXIDATION OF ALPHA-KETO ACIDS[1]

KENNETH W. COCHRAN[2] AND KENNETH P. DUBOIS

U. S. Air Force Radiation Laboratory and the Department of Pharmacology, The University of Chicago, Chicago, Illinois

RECENT observations in this laboratory (DuBois, Cochran and Doull, 1951; DuBois, Cochran and Zerwic, 1951) suggested that some of the sex hormones and adrenal cortical hormones exert a regulatory action on citric acid synthesis in the liver *in vitro*. By the use of the sequential blocking technique of Potter (1951) in which the oxidative disposal of citric acid is inhibited (Buffa and Peters, 1949, 1950; Liebecq and Peters, 1949; Elliott and Kalnitsky, 1950; Potter and Busch, 1950) by the administration of fluoroacetate it was found (DuBois, Cochran and Doull, 1951) that the livers of female rats accumulated large quantities of citric acid whereas male rats are unable to accumulate citric acid in the liver following fluoroacetate treatment (Potter and Busch, 1950; Potter, Busch and Bothwell, 1950). Evidence implicating steroid hormones in the sex difference to fluoroacetate was obtained from the observations (DuBois, Cochran, and Zerwic, 1951) that after castration, administration of estradiol, or adrenalectomy male rats acquired the ability to accumulate citric acid in the liver and treatment of female rats with testosterone decreased citrate accumulation in the liver following fluoroacetate treatment. In contrast to the inability of fluoroacetate-treated male rats to accumulate citric acid in the liver *in vivo* after fluoroacetate treatment, homogenates of liver from male rats are capable of forming large quantities of citric acid *in vitro* (Potter and Busch, 1950; Pardee and Potter, 1949a) which suggests that some mechanism inhibiting citric acid formation in the intact animals is lost when the tissue is homogenized. The observations described above stimulated our interest in ascertaining whether the addition of various steroids to liver homogenates *in vitro* would inhibit citrate synthesis. The present communication describes the results of experiments which indicate that citrate formation and the oxidation of pyruvate and alpha-ketoglutarate are in-

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[2] Present address: Department of Epidemiology and Virus Laboratory, School of Public Health, University of Michigan, Ann Arbor, Michigan.

hibited by testosterone, desoxycorticosterone, ethinyl estradiol, progesterone and diethylstilbestrol and to a lesser extent by some other steroids. Since the appearance of these results in abstract form (Cochran, Zerwic and DuBois, 1951; Cochran and Dubois, 1952),Grant and Taylor (1953) have reported similar findings with some of the steroids.

MATERIALS AND METHODS

Male and female Sprague-Dawley rats weighing between 200 and 350 gm., hybrid guinea pigs, 350–600 gm., and CF-1 mice, 20–30 gm., were used for these studies. The oxidation of various intermediates of the tricarboxylic acid cycle was studied using a conventional Warburg apparatus and the test system of Pardee and Potter (1949a). All substrates were employed at a final concentration of 0.0053 M except that in the formation of acetoacetate a final concentration of 0.0035 M pyruvate was used. In those instances where malonate was employed a final concentration of 0.004 M was used in the test system. The amounts of tissue used per Warburg flask were: liver, 50 mg.; kidney, 30 mg.; and brain, 50 mg. The tissues were added as 10% isotonic potassium chloride homogenates. Oxygen consumption was measured during the last 30 min. of the 40-min. incubation period. At the end of the incubation period the flasks were promptly cooled in crushed ice and 2 ml. of 17.5% trichloroacetic acid were added. After centrifuging, aliquots were analyzed for citrate or alpha-ketoglutarate. In those experiments where acetoacetate formation was measured, the addition of trichloroacetic acid to the Warburg flasks was omitted and 0.5 ml. aliquots of the flask contents were withdrawn and added to centrifuge tubes containing equal volumes of trichloroacetic acid and analyzed for pyruvate. The remainder of the flask contents was assayed for acetoacetate by the manometric method (Umbreit, Burris and Stauffer, 1949). Citrate was measured by the method of Natelson, Lugovoy and Pincus (1947) as modified by Potter and Busch (1950). Pyruvate and alpha-ketoglutarate were analyzed by the method of Friedemann and Haugen (1943). Alpha-ketoglutaric acid oxidase activity was measured by the procedure of Ackermann (1950) with the addition of 0.067 M potassium chloride to the system. Crystalline steroids[3] were used throughout these experiments. The hormones were dissolved in ethanol and 0.02 ml. of an ethanol solution was added per flask with appropriate ethanol controls being included in each experiment.

RESULTS

Inhibitory action of steroids on citrate synthesis by tissue homogenates. To ascertain whether an inhibition of citrate synthesis could be demonstrated *in vitro* measurements of the influence of several steroids on citrate formation by liver homogenates were made. Pyruvate and fumarate were used as substrates in the test system developed by Pardee and Potter (1949a) and measurements were made of both oxygen consumption and citrate formation. The average normal oxygen consumption by rat liver homogenates was 7.2 (3.3–9.4) μM per 50 mg. of wet tissue per 40 min. and the average amount of citrate formed during this period of time was 3.4 (2.1–5.0) μM per 50 mg. The addition of the maximum amount of ethanol

[3] Kindly supplied by the Schering Corporation, Bloomfield, New Jersey.

(0.02 ml.) employed as a solvent for the steroids did not significantly affect oxygen consumption or citrate formation. Data on the effects of several steroids on oxygen consumption and citric acid formation are shown in Table 1 in which the values are presented both as μM of oxygen and citrate formed per 50 mg. of tissue per 40 min. and as the per cent inhibition with respect to the ethanol controls.

TABLE 1. EFFECT OF STEROIDS ON OXYGEN CONSUMPTION AND CITRATE FORMATION BY RAT LIVER HOMOGENATES *in vitro*

Pyruvate and fumarate (each 0.0053 M)

Steroid		μg. steroid/Warburg flask						
		μM/50 mg. of tissue/40′				Av. % inhibition		
		Control	100	150	200	100	150	200
Diethylstilbestrol	O_2 consumption	6.1, 15.9	3.4, 5.3	0.7, 0.6	0, 0.3	55	92	99
	Citrate formation	6.0, 7.9	2.7, 2.7	0.9, 0.6	1.1, 1.1	60	88	84
Progesterone	O_2	16.7, 15.0	7.6, 6.5	3.5, 3.8	2.3, 2.1	56	77	86
	Citrate	8.8, 7.7	3.5, 1.9	2.5, 1.5	1.9, 1.0	68	76	82
Ethinyl estradiol	O_2	9.1, 4.8	3.2, 3.9	1.1, 2.5	0.9, 1.2	42	68	82
	Citrate	2.9, 3.2	1.4, 1.6	0.5, 1.0	0.4, 1.4	50	75	71
Desoxycorticosterone	O_2	15.6, 12.5	13.0, 14.0	7.0, 6.8	4.4, 5.3	2	50	65
	Citrate	7.0, 6.6	3.0, 4.6	2.1, 2.4	1.7, 1.9	43	67	74
Testosterone	O_2	17.5, 13.2	16.2, 10.9	10.5, 9.0	8.9, 5.9	12	36	52
	Citrate	8.9, 8.2	6.8, 4.6	5.7, 3.8	4.6, 4.0	34	45	50
Estradiol	O_2	8.3, 12.8	6.7, 12.9	7.0, 10.9	6.1, 10.3	9	16	23
	Citrate	5.2, 5.5	4.2, 5.0	3.9, 4.6	3.9, 4.0	14	20	26

The data in Table 1 show that several steroids cause a marked inhibition of both oxygen consumption and citrate formation. Thus, diethylstilbestrol, progesterone, ethinyl estradiol, desoxycorticosterone, and testosterone were all effective inhibitors. The approximate molar concentrations of these steroids required to produce 50% inhibition of both oxygen consumption and citrate formation were: diethylstilbestrol, 9×10^{-5}; progesterone, 5×10^{-5}; ethinyl estradiol, 1×10^{-4}; desoxycorticosterone, 1.1×10^{-4}; and testosterone, 2.3×10^{-4}. Although the control values for different liver samples varied considerably the percentage inhibition by a given amount of steroid was remarkably constant from one experiment to another. Several other steroids were tested under the same conditions and found to be less effective inhibitors. These included estradiol, pregnenolone, estrone, methyl androstanediol, methyl androstenediol, cortisone, cholesterol and ouabain. Thus 200 μg. of estradiol per flask caused only 23% inhibition of oxygen consumption and 26% inhibition of citrate formation, while with pregnenolone the values were 14 and 34% inhibited respectively, and estrone caused 14% inhibition of oxygen consumption and 13% inhibition of citrate formation. The other steroids tested produced no significant alteration in citrate formation or oxygen consumption. Inhibition of citrate synthesis by testosterone, progesterone, desoxycorticosterone and lack of inhibition by cortisone have been confirmed by Grant and Taylor (1953). The results of our experiments are also in agreement

with the observations of Guidry, Segaloff and Altschul (1952) who found that estradiol and estrone failed to inhibit the oxidative capacity of liver homogenates using the oxidative phosphorylation system of Pardee and Potter (1949a) with pyruvate, furmarate, and citrate as substrates. However, Guidry *et al.* (1952) observed that estradiol and estrone inhibit the oxidation of other substrates such as lactate, glutamate, glycerophosphate and isocitrate. In our experiments the order of effectiveness of all the steriods in inhibiting citrate synthesis is approximately the same as that found by Gordan and Elliott (1947) and by Hayano, Schiller and Dorfman (1950) in studies on the effects of these compounds on other oxidative reactions. The results of our *in vitro* experiments with testosterone and desoxycorticosterone are in agreement with previous experiments in this laboratory (DuBois, Cochran and Zerwic, 1951) which suggested that testosterone and one or more adrenal hormones inhibit citrate synthesis in the liver *in vivo*

Other investigators (Gordan and Elliott, 1947; Eisenberg, Gordan and Elliott, 1949a; Hayano, Schiller and Dorfman, 1950) have observed that testosterone inhibits the respiration of rat brain, skeletal muscle and kidney. It was, therefore, of interest to examine the effect of steroids on citrate formation *in vitro* by tissues other than liver. In our *in vitro* studies the average oxygen consumption by rat kidney homogenates in the presence of 0.02 ml. of ethanol was 20.0 (17.0–22.9) μM per 50 mg. of tissue per 40 min. and the average citrate formation was 2.5 (1.9–4.0) μM. The corresponding values for rat brain were 4.9 (3.9–5.7) and 1.0 (0.7–1.4) for oxygen consumption and citrate formation respectively. The data in Table 2 show the per cent inhibition of oxygen utilization and citrate formation when various steroids were added at a level of 200 μg. per Warburg flask to kidney and brain homogenates. Testosterone, desoxycorticosterone, progesterone and diethylstilbestrol, which were all effective inhibitors of citrate synthesis by liver homogenates, also inhibited synthesis of citric acid by brain and kidney homogenates. From a comparison of the action of the steroids on these three tissues it may be noted that citrate synthesis by kidney was more sensitive than liver to inhibition by the steroids *in vitro* while brain was the least sensitive. Thus, 200 μg. per flask of desoxycorticosterone inhibited oxygen uptake and citrate formation by liver 65 and 74% respectively while kidney was 82 and 83% inhibited and the oxygen consumption and citrate formation by brain was 36 and 46% inhibited respectively. Estradiol, methyl androstenediol, estrone, cortisone, cholesterol and ouabain were relatively ineffective inhibitors of oxygen consumption and citric acid formation by brain and kidney homogenates as was the case with liver homogenates.

In view of the high sensitivity of the respiration of kidney toward inhibi-

TABLE 2. EFFECT OF STEROIDS ON OXYGEN CONSUMPTION AND CITRATE FORMATION BY RAT KIDNEY AND BRAIN HOMOGENATES *in vitro*

Pyruvate and fumarate (each 0.0053 M)

Steroid, 200 μg./Warburg flask		% inhibition	
		Kidney	Brain
Diethylstilbestrol	O_2 consumption	100 (100, 100)*	94 (88, 100)
	Citrate formation	100 (99, 100)	65 (59, 71)
Progesterone	O_2	96 (95, 96)	74 (74, 75)
	Citrate	100 (99, 100)	62 (55, 68)
Desoxycorticosterone	O_2	81 (73, 89)	36 (26, 52)
	Citrate	83 (79, 87)	46 (35, 57)
Testosterone	O_2	59 (47–71)	25 (5, 57)
	Citrate	50 (36–69)	46 (28–64)
Estradiol	O_2	32 (27, 37)	22 (17, 27)
	Citrate	26 (26, 26)	16 (10, 21)

* Numbers in parentheses are values for separate experiments or ranges of values for several experiments.

tion by diethylstilbestrol and progesterone additional experiments were performed using lower concentrations of these compounds. A concentration of 50 μg. of diethylstilbestrol per flask caused 88 and 79% inhibition of oxygen consumption and citrate formation by rat kidney homogenates and produced 18% inhibition of oxygen consumption and 36% inhibition of citrate formation by liver homogenates. Progesterone at a concentration of 50 μg. per flask caused 61% inhibition of the oxygen consumption and 48% inhibition of citrate formation by rat kidney homogenates while oxygen consumption by rat liver was inhibited 58% and citrate formation was inhibited 54%. Kalnitsky (1948) found that the presence of fluoroacetate increased the formation of citrate from oxalacetate by rabbit kidney cortex homogenates. The addition of 0.01 M fluoroacetate to kidney homogenates in the system of Pardee and Potter (1949a) which we employed decreased the oxygen consumption and increased citrate formation but did not influence the inhibitory action of steroids. The addition of similar concentrations of fluoroacetate did not significantly affect the oxygen consumption or citrate formation by brain homogenates. In the case of liver there was a decrease of approximately 50% in both oxygen consumption and citrate formation but in no instance was the inhibitory effect of the steroids changed.

Since testosterone has been shown to inhibit tissue respiration in species other than the rat (Dirscherl and Knüchel, 1950; Kochakian, 1951) measurements were made of the inhibitory effects of steroids on citrate formation *in vitro* using tissues from mice and guinea pigs. Citrate formation *in vitro* by homogenates of liver from mice and guinea pigs was inhibited by

diethylstilbestrol, progesterone, desoxycorticosterone and testosterone to the same degree as was observed with rat tissues.

Influence of steroids on acetoacetate formation by liver homogenates. Under the conditions of the experiments described above the entire citric acid cycle is operative (Pardee and Potter, 1949a). Thus, the decrease in oxygen consumption and citrate formation by steroids might be the result of inhibition of one or more of the steps in the oxidative phase of carbohydrate metabolism. In an attempt to locate the specific site of the inhibitory action of these steroids we undertook studies on several individual oxidative reactions. Consideration was given to the possibility that the inhibitory action occurred at the stage of pyruvate metabolism. After conversion of pyruvate to a two-carbon fragment it may combine with oxalacetate to form citrate. In liver another important pathway is available whereby condensation of a pair of two-carbon fragments may occur resulting in the formation of acetoacetate. Lehninger (1946) demonstrated that pyruvate is quantitatively converted by liver preparations to acetoacetate when malonate is present in the system. Inhibition of both the formation of citrate and acetoacetate would indicate that steroids inhibit either some stage of acetyl-coenzyme A metabolism or the oxidative decarboxylation of pyruvate.

The possibility that the steroids interfere directly with acetate metabolism is unlikely because we observed no effect by testosterone on the acetylation of sulfanilamide *in vivo*. For these experiments groups each containing four male and female rats were given 2.5 mg./kg. of testosterone propionate daily for 14 days and were then given 100 mg./kg. of sulfanilamide at hourly intervals for 3 hours. Normal male and female rats which received no testosterone were given the same quantity of sulfanilamide and the urinary excretion of acetylated sulfanilamide was measured. Both the control and testosterone-treated groups excreted approximately 30% of the administered sulfanilamide in the acetylated form indicating that testosterone exerts no influence on this acetylation reaction.

Acetoacetate production by rat liver homogenates was measured using the test system of Pardee and Potter (1949a) with pyruvate as the substrate and with malonate added (Lehninger, 1946) to block the citric acid cycle. The reaction was followed by measuring oxygen consumption, acetoacetate formation and pyruvate utilization. In the presence of 0.004 M malonate the average values for 50 mg. of liver per 40 min. were 2.0, (1.6–3.6) μM of oxygen consumed, 2.1 (1.1–4.3) μM of acetoacetate formed and 4.6 (2.0–7.7) μM of pyruvate utilized. Data on the effects of several steroids on oxygen consumption, acetoacetate formation and pyruvate utilization are presented in Table 3 in which values for two separate experiments with each steroid are presented. These data show that testos-

terone, desoxycorticosterone, progesterone and diethylstilbestrol inhibit the oxidation of pyruvate by rat liver homogenates. Estradiol, methyl androstenediol, estrone, pregnenolone, cortisone and cholesterol were found to be relatively ineffective as inhibitors. The utilization of pyruvate by this system and the formation of acetoacetate were inhibited to a similar extent by these steroids. The amount of inhibition was dependent upon the concentration steroid in the test system and resembled quantitatively the inhibition of oxygen consumption and citrate formation using pyruvate and fumarate as substrates. This inhibition of the formation of

TABLE 3. EFFECT OF STEROIDS ON OXYGEN CONSUMPTION, ACETOACETATE FORMATION AND PYRUVATE UTILIZATION BY RAT LIVER HOMOGENATES *in vitro*

Pyruvate, 0.0035 M

Steroid		μg. steroid/Warburg flask						
		μM/50 mg. of tissue/40′				Av. % inhibition		
		Control	100	150	200	100	150	200
Diethylstilbestrol	O_2 consumption	3.8, 3.8	3.7, 1.8	0.1, 0.4	0, 0.4	28	93	94
	Acetoacetate formation	4.9, 3.6	1.6, 0.8	0.4, 0.3	0.4, 0.3	72	92	92
	Pyruvate utilization	9.5, 7.6	7.1, 5.6	1.2, 2.5	4.7, 1.5	26	77	65
Progesterone	O_2 consumption	4.3, 2.2	2.5, 2.4	1.5, 0.4	0.3, 0.4	16	74	88
	Acetoacetate formation	3.8, 2.5	1.4, 0.8	1.7, 0.8	0.9, 0.58	64	60	76
	Pyruvate utilization	9.9, 4.6	5.6, 3.0	2.8, 1.6	2.4, 0	38	69	88
Desoxycorticosterone	O_2 consumption	7.2, 3.3	4.7, 3.0	4.5, 2.2	3.4, 0.7	22	26	66
	Acetoacetate formation	8.6, 4.0	5.8, 2.4	4.8, 1.2	3.3, 1.5	36	58	62
	Pyruvate utilization	22.0, 11.0	19.4, 6.6	16.4, 4.8	14.2, 4.2	26	46	48
Testosterone	O_2 consumption	3.7, 3.7	2.6, 2.6	2.6, 1.8	1.9 —	30	40	—
	Acetoacetate	3.1, 5.0	1.3, 3.7	0.6, 3.2	0.9 —	42	42	—
	Pyruvate	— 9.2	— 6.6	— 6.6	— —	28	28	—
Estradiol	O_2 consumption	4.3, 3.3	— —	2.7, 3.2	5.3, 2.1	—	20	30
	Acetoacetate	4.7, 3.2	— —	2.6, 3.2	3.1, 2.5	—	22	28
	Pyruvate	9.4, 5.0	— —	5.4, 5.0	7.1, 3.8	—	21	24

acetoacetate from pyruvate by certain steroids is in agreement with the previous report of the inhibition of pyruvate oxidation by testosterone (Eisenberg, Gordan and Elliott, 1949b).

Effect of steroids on the oxidation of alpha-ketoglutarate. In view of the ability of certain steroids to inhibit pyruvate oxidation we were interested in determining whether the oxidation of alpha-ketoglutarate was also inhibited by these compounds since the enzymatic mechanism for the oxidative decarboxylation of these keto-acids is similar (Ochoa, 1951). The steroids employed for these tests included those compounds which were the most effective inhibitors of citrate and acetoacetate formation. They included testosterone, desoxycorticosterone, progesterone, ethinyl estradiol and diethylstilbestrol. Measurements were made of both oxygen consumption and alpha-ketoglutarate utilization. The average normal value for oxygen uptake was 5.6 (4.4–8.3) μM and for alpha-ketoglutarate utilization was 12.2 (9.8–15.5) μM per 50 mg. of liver per 40 min. Data on the inhibition of oxygen uptake and alpha-ketoglutarate utilization produced

by these steroids are given in Table 4 in which the results of two experiments with each concentration of the steroids are presented. These data indicate that the steroids which were effective inhibitors of citrate and acetoacetate formation are similarly effective as inhibitors of alpha-ketoglutarate oxidation.

To ascertain whether the inhibition was due to a reaction between an essential cofactor for alpha-ketoglutarate oxidation various components of the test system were omitted or increased to five times the usual quantity. Omission or a five-fold increase in the quantities of the various reactants necessary for maximum activity of alpha-ketoglutaric acid oxidase

TABLE 4. EFFECT OF STEROIDS ON THE OXIDATION OF ALPHA-KETOGLUTARATE BY RAT LIVER HOMOGENATES *in vitro*

Alpha-ketoglutarate, 0.0053 M

Steroid		μg. steroid/Warburg flask						
		μM/50 mg. of tissue/40′				Av. % inhibition		
		Control	100	150	200	100	150	200
Diethylstilbestrol	O_2	4.9, 5.8	2.7, 1.8	0.7, 0.2	0.2, 0.1	57	92	98
	α-KG	11.0, 14.3	5.2, 4.6	1.5, 0.3	0.2, 0	60	92	99
Ethinyl estradiol	O_2	5.9, 6.2	4.8, 3.9	0.9, 1.9	0.7, 1.1	28	77	85
	α-KG	14.0, 9.8	10.8, 7.6	1.7, 3.1	0.8, 0.2	22	78	86
Progesterone	O_2	4.8, 5.2	3.9, 3.8	3.5, 1.8	1.0, 0.4	23	46	86
	α-KG	13.2, 12.4	11.6, 9.6	7.9, 7.1	3.2, 2.7	18	42	77
Desoxycorticosterone	O_2	6.2, 5.8	5.5, 4.3	2.8, 3.3	2.2, 2.3	18	49	62
	α-KG	13.5, 11.6	13.9, 10.3	9.9, 7.6	8.1, 5.2	4	30	48
Testosterone	O_2	3.6, 4.4	3.5, 4.5	2.4, 3.0	1.5, 2.1	0	32	55
	α-KG	9.1, 11.2	6.8, 9.0	4.5, 6.9	2.7, 4.4	22	44	66

as described by Ackermann (1950) failed to alter the percentage inhibition when progesterone was added to the system. Thus, the inhibition of this reaction by steroids does not appear to be due to interference with known essential cofactors.

Effect of steroids on the oxidation of succinate. The effects of steroids on the oxidation of succinate *in vitro* were also studied. It has been observed (McShan and Meyer, 1946; Case and Dickens, 1948; Kalman, 1952) that the oxidation of succinate is inhibited by various steroids through an action either on the succinoxidase or cytochrome oxidase systems. McShan, Meyer and Erway (1947) found that androgens did not inhibit the succinoxidase system of rat liver and Eisenberg, Gordan and Elliott (1949b) found that succinate oxidation was not inhibited by amounts of testosterone which inhibited the oxidation of glucose. Other studies (Guidry, Segaloff and Altschul, 1952; Sourkes and Heneage, 1952) have indicated that the oxidation of succinate is more resistant to inhibition than is the oxidation of certain other substrates. In the present studies when succinate was used as the substrate in the test system of Pardee and Potter (1949a)

the oxygen consumption was 6.3 μM per 50 mg. of liver per 40 min. The steroids tested were limited to those found to be effective inhibitors of alpha-ketoglutarate oxidation and of citrate and acetoacetate formation. Whereas diethylstilbestrol at a concentration of 200 μg. per flask produced almost complete inhibition of citrate and acetoacetate formation as well as alpha-ketoglutarate oxidation, it caused only 5% inhibition of succcinate oxidation. Progesterone at the same concentration caused 19% inhibition of succinate oxidation, compared with approximately 90% inhibition of alpha-ketoglutarate oxidation and citrate and acetoacetate formation. Testosterone and desoxycorticosterone failed to produce any inhibition of succinate oxidation whereas these compounds caused approximately 50 and 65% inhibition respectively of the other reactions. Since the reactions which were strongly inhibited involve oxidative decarboxylation it appears that the oxidation of alpha-keto acids is especially susceptible to inhibition by some steroid hormones.

DISCUSSION

During the course of investigations on the effects of x-irradiation on intermediary carbohydrate metabolism a sex difference in citrate synthesis in the livers of rats *in vivo* was observed (DuBois, Cochran and Doull, 1951). Whereas no significant citrate accumulation occurred in the livers of fluoroacetate-poisoned male rats in agreement with previous observations (Potter and Busch, 1950; Potter, Busch and Bothwell, 1951) female rats accumulated large quantities of citrate in the liver. The ability of male rats to accumulate citrate in the liver to approximately the same degree as females following x-irradiation (DuBois, Cochran and Doull, 1951), castration, adenalectomy, and treatment with estradiol (DuBois, Cochran and Zerwic, 1951) as well as the reduction in citrate accumulation in the livers of females following testosterone treatment suggested that testosterone and one or more of the adrenal hormones normally imposed some restraint on citrate formation in the liver *in vivo*. In support of these previous findings the present investigation has provided data showing that testosterone and desoxycorticosterone inhibit the formation of citric acid *in vitro*. Certain other steroids were also found to be effective inhibitors of citrate synthesis by liver tissue *in vitro* including progesterone, ethinyl estradiol and diethylstilbestrol. Similar results were obtained *in vitro* with liver from several species and with kidney and brain from rats. Further *in vitro* studies revealed that those steroids which effectively inhibit the synthesis of citrate from pyruvate and fumarate also inhibit the principal alternative pathway for pyruvate metabolism which leads to the formation of acetoacetic acid by the liver. Studies on the effect of steroids on the oxidation of other members of the tricarboxylic acid cycle revealed that

the oxidation of alpha-ketoglutarate was inhibited by the same steroids and to the same extent as was the formation of citrate from pyruvate and fumarate and the formation of acetoacetate from pyruvate. In contrast, the oxidation of succinate was not appreciably inhibited by these compounds.

In a recent review Lieberman and Teich (1953) discuss some mechanisms by which steroid hormones may act on enzyme systems *in vitro*. Inhibition of cytochrome oxidase has been suggested (McShan and Meyer, 1946) but the results of other studies (Eisenberg, Gordan and Elliott, 1949b; Hayano, Schiller and Dorfman, 1950; Guidry, Segaloff and Altschul, 1952) and the present investigation do not indicate that cytochrome-linked dehydrogenations are as susceptible to inhibition by steroids as are certain other oxidative systems. Guidry, Segaloff and Altschul (1952) suggested that steroid inhibition is specific for oxidations linked by coenzymes I and II. It may be noted, however, that in our investigation varying the coenzyme I concentration failed to alter the inhibition of alpha-ketoglutaric acid oxidase by progesterone. Similar results were obtained by Grant and Taylor (1953). The results of experiments described in this communication indicate that one locus for an inhibitory action by certain steroids *in vitro* is at the stage of the oxidative decarboxylation of alpha-keto acids.

SUMMARY

The syntheis of citrate from pyruvate and fumarate by rat liver homogenates was inhibited *in vitro* by testosterone, desoxycorticosterone, ethinyl estradiol, progesterone and diethylstilbestrol. Other steroids which were relatively ineffective as inhibitors of citate synthesis *in vitro* were estradiol, estrone, pregnenolone, methyl androstenediol, methyl androstanediol, cortisone, cholesterol and ouabain.

The synthesis of acetoacetate from pyruvate was markedly inhibited by testosterone, desoxycorticosterone, progesterone and diethylstilbestrol. Those steroids which inhibited the synthesis of citrate and acetoacetate also inhibited the oxidation of alpha-ketoglutarate *in vitro*. The oxidation of succinate was not inhibited *in vitro* by those steroids which inhibited the oxidation of alpha-ketoglutarate and the synthesis of citrate and acetoacetate.

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THE EFFECT OF CORTISONE ON THE COLLAGEN AND HEXOSAMINE CONTENT OF THE SKIN AND FEMURS OF ONE YEAR OLD RATS

HARRY SOBEL AND JESSIE MARMORSTON

Department of Biochemistry, Division of Laboratories and the Institute for Medical Research, Cedars of Lebanon Hospital, Los Angeles, California

OSTEOPOROSIS and striae formation of the skin of patients with Cushing's syndrome is generally thought to be related to an overproduction of the adrenal cortical gluconeogenic hormones (Albright and Reifenstein, 1948). This would result in a net loss of protein from the bones and skin. Since collagen is the predominant protein of these organs it is implied that a loss of this protein is involved in the pathogenesis of these lesions. These considerations seem to be in conflict with the recent observations that collagen is relatively inert metabolically (Neuberger, Perrone and Slack, 1951; Robertson, 1952).

The collagen and hexosamine contents of the skins (Sobel, Zutrauen, and Marmorston, 1953) and femurs (Sobel and Marmorston, to be reported) of young growing rats have been investigated. The quantity of collagen deposited in these organs is a function of the maximum weight attained by the animal and does not appear to be influenced by a variety of experimental situations. In this investigation cortisone was given to one year old rats at dose levels sufficient to induce large losses in weight and the collagen and hexosamine contents of the skin and femurs were studied. Hexosamine determinations were made since this substance is a component of the mucopolysaccharides and mucoproteins of these organs.

EXPERIMENTAL

One year old rats of the Slonaker-Addis strain were used in these experiments and, except where indicated, were maintained on stock diet (Addis and Gray, 1950). Synthetic diets contained 8% and 60% casein, 8% fat, 1% defatted liver powder, an adequate supply of minerals and vitamins, and glucose to make up the bulk of the diet. Cortisone acetate suspension (Cortone) was administered subcutaneously. Semi-starvation was induced in some rats by limiting food intake to 5 gm. of stock diet daily. Others were kept in a cold room at 4° C. Thyroxine was administered subcutaneously in doses of 500 μg. daily.

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The procedure for determining the quantity of collagen and hexosamine in skin has been reported (Sobel, Zutrauen and Marmorston, 1953). The procedure for femurs will be reported elsewhere. Briefly, the skin, except that of the face, paws and tail is removed, scraped free of adhering muscle, extracted with ether and acetone to remove fat and the collagen is converted into gelatin by autoclaving with water. The protein content of the extract is determined by the biuret procedure (Kingsley, 1939). Hexosamine of the extract is determined by a modified Elson-Morgan procedure (Johnston, Ogston and Stanier, 1951) on acid hydrolysates of the gelatin extract and the water insoluble residue. The value is given as the sum of the quantity present in each fraction. Both femurs are cleaned of all adhering tissue and extracted with ether and acetone. The lengths are carefully measured with a vernier caliper. One femur is weighed, demineralized with 10% trichloroacetic acid solution and acetone dried. The organic residue is weighed to determine the mineral content by difference and then autoclaved with water. The protein content of the extract is determined. The other femur is hydrolyzed directly and the hexosamine content of the hydrolysate is determined. The marrow contributes only a few per cent to the total hexosamine. The data from male and female rats were pooled since the quantity of collagen and hexosamine in skin and femurs was previously found to be a function of body weight and no sex differences have been found.

RESULTS

In order to interpret the observations obtained in experiments of this type it is necessary to relate the quantity of the constituent in skin and bone to the size of the animal (Sobel, Zutrauen and Marmorston, 1953). For this purpose regression equations must be derived relating the quantity of the constituent in question to an independent variable which is a function of the size of the rat. Since body weight in one year old rats could be greatly disturbed by variations in the content of fat and water it was considered unsuitable as a basis for comparison. It is well known that femur length is a function of maximum growth of the animal and that the length does not diminish (in the absence of distortion) in the face of large weight losses such as were induced in this investigation. Accordingly regressions (Table 1) were derived relating collagen, hexosamine and mineral contents of the femur, and the collagen and hexosamine contents of the skin to the femur length of normal one year old rats. A regression equation correlating femur length and body weight was also derived. These equations were all based on the observations that an arithmetical linear relationship appears to exist between femur length and the dependent variable in the year old rats used in this study. *They do not apply if younger animals*

TABLE 1. REGRESSION EQUATIONS FOR NORMAL ONE YEAR OLD RATS RELATING COMBINED FEMUR LENGTHS (L) WITH SKIN AND COMBINED FEMUR COLLAGEN (C), SKIN AND COMBINED FEMUR HEXOSAMINE (H) AND COMBINED FEMUR MINERAL CONTENT (I).

W = body weight. N = no. of cases, r, V_e, $\bar{x}$, SSx = see footnote

Body Weight				
(1) $W \times 10^{-2} = -8.237 + 1.743L$				
$r = .872$	$V_e = .1019$	$n = 43$	$\bar{x} = 6.647$	$SSx = 4.409$
Skin				
(2) $C = -26.52 + 5.01L$				
$r = .923$	$V_e = 4.22$	$n = 34$	$\bar{x} = 6.605$	$SSx = 5.383$
(3) $H = -107.75 + 20.73L$				
$r = .907$	$V_e = 24.04$	$n = 31$	$\bar{x} = 6.626$	$SSx = 5.312$
Bone				
(4) $C = -316.05 + 73.88L$				
$r = .907$	$V_e = 156$	$n = 29$	$\bar{x} = 6.570$	$SSx = 3.630$
(5) $H = -4.01 + 1.11L$				
$r = .882$	$V_e = .073564$	$n = 35$	$\bar{x} = 6.569$	$SSx = 4.0340$
(6) $I = -1309.45 + 300.16L$				
$r = .938$	$V_e = 1433.94$	$n = 37$	$\bar{x} = 6.621$	$SSx = 4.068$

$$y' = a + bx; \quad a = \bar{y} - b\bar{x}; \quad \bar{y} = \frac{\sum y}{n}; \quad \bar{x} = \frac{\sum x}{n}; \quad b = \frac{k}{SSx}$$

$$k = (\sum xy - \bar{x} \sum y); \qquad SS_x = \sum x^2 - \frac{(\sum x)^2}{n}$$

$$r = \text{correlation} = \frac{k}{\sqrt{SS_x SS_y}}; \quad SS_y = \sum y^2 - \frac{(\sum y)^2}{n}$$

$$Ve = SS_{e/N}; \quad N = n - 2; \quad SS_e = SS_y - SS_r; \quad SS_r = kb$$

are included. Justification in the use of an arithmetical linear relationship is found in the satisfactory correlation coefficients (r).

Further justification for the use of bone length as a basis for comparison with the normal can be seen in Table 2 where the results on observations of the experimentally treated animals are recorded. As a result of the treatments (cortisone and thyroxine administration, semi-starvation and exposure to cold) very large losses in weight were observed. Nevertheless the body weight for almost every group at the start of each experiment was found not to differ significantly from the value predicted by Equation 1 using combined femur length measured at the completion of the experiment. In only two groups (11 and 14) were statistically important differences observed. In evaluating the likelihood that these two differences could have been due to random chance, and therefore be considered not significant, one should remember that a probability of .05 is expected to be found with one sample if twenty random samples are drawn. The present series is composed of sixteen samples. In every instance the predicted body weight exceeded by a considerable quantity the observed weights at the completion of the experiments. In most instances these differences were extremely high statistically.

Effect of Treatment upon the Collagen Content of the Skin.—In none of the

TABLE 2. RESULTS OF ANALYSES OF SKINS AND FEMURS OF ONE YEAR OLD RATS RECEIVING CORTISONE ACETATE, THYROXINE, SUBJECTED TO SEMI-STARVATION AND EXPOSED TO COLD

Group No.	Treatment	Duration, days	No. of rats	Starting weight		Final weight		Skin				Femurs					
				Gm.	t_1	Gm.	t_1	Total collagen (gm.)	t_2	Total hexosamine (mg.)	t_3	Length (both femurs) cm.	Collagen (both femurs) mg.	t_4	Hexosamine (both femurs) mg.	t_5	Inorganic (both femurs) mg.	t_6
1	15–18 mg./kilo cortisone acetate	53–54	5 ♂	385.0	+1.17	262.8	− 7.23	8.24 (4)	+0.51	31.76	−0.96	6.834	207.2	+3.22	3.364	−1.71	741.2	−0.035
2	20 mg./kilo cortisone acetate	19–20	4 ♂	371.5	+0.53	260.0	− 6.23	7.41	−0.45	26.23	−3.70	6.865	181.5	−1.48	2.927	−4.97	700.0	−2.65
3	20 mg./kilo cortisone acetate	19	5 ♀	258.2	−1.37	207.6	− 4.34	4.11 (4)	−1.00	15.27	−3.17	6.322	142.6	−1.46	2.435	−4.48	568.6	−1.17
4	30 mg./kilo cortisone acetate	15–27 (19)	4 ♂	452.5	+1.77	296.0	− 7.70	7.82	−1.61	33.40	−2.81	7.155	221.7	+1.93	3.340	−4.13	851.5	+0.67
5	30 mg./kilo cortisone acetate	—	6 ♂	395.3	+0.76	268.7	− 8.77	—	—	—	—	6.936	—	—	3.020	−5.84	—	—
6	30 mg./kilo cortisone acetate	21–34 (26)	6 ♂	380.8	−1.09	237.5	−11.83	6.57 (5)	−1.73	30.79	−3.13	6.994	199.2	−0.28	3.304	−3.92	754.0	−2.45
7	30 mg./kilo cortisone acetate +8% dietary protein	16–21 (19)	6 ♂	378.7	−0.02	249.6	− 9.58	7.71	−0.39	38.15	+1.40	6.900	205.6	+2.21	3.300	−3.06	772.6	+0.70
8	30 mg./kilo cortisone acetate +60% dietary protein	21–33 (27)	5 ♂	368.6	−0.47	249.2	− 8.65	6.88	−1.63	39.14	+0.97	6.980	205.6	+0.97	3.286	−3.63	703.0	+1.00
9	50 mg./kilo cortisone acetate	7–13 (11)	6 ♂	393.5	−1.96	281.1	−10.45	7.67 (5)	−1.73	29.75	−5.12	7.155	211.1	−0.29	3.362	−4.84	792.0	−2.84
10	Semi-starvation	14–20 (18)	4 ♂	292.5	−0.58	147.3	− 9.52	5.34	−0.47	—	—	6.458	162.0	+0.14	2.968	−1.39	615.5	−0.70
11	Semi-starvation	21	5 ♂	378.6	−2.37	251	−11.70	7.38	−1.76	30.00	−4.18	7.100	204.3	−0.72	3.090	−6.13	766.4	−3.13
12	Thyroxine administration	22–28 (26)	4 ♂	408	−0.04	287.7	− 7.37	4.48	−0.40	33.90	−2.19	7.070	213.6	+1.12	3.245	−4.17	786.0	−1.36
13	Thyroxine administration	20–30 (27)	4 ♀	283	+0.20	198	− 4.98	4.54	−0.62	22.25	−0.48	6.330	150.3	−0.21	2.760	−1.85	551.0	−2.04
14	Cold room	8–11 (10)	4 ♂	438.7	+3.07	338	− 3.82	8.97	+0.48	39.85	+1.11	6.985	190.0	−1.54	3.467	−1.97	808.5	+1.10
15	Cold room	6–13 (8)	4 ♀	271	+0.36	239	− 2.26	4.18	−0.84	22.28	−0.31	6.310	138.5 (5)	−1.82	2.955 (5)	−0.28	622 (5)	+1.93
16	Cold room	12–44 (23)	6 ♂	399.3	+0.65	280	− 6.65	8.01	−0.38	44.76	+2.10	6.956	199.0	+0.96	3.472	−1.91	802.0	+1.48

$$t = \frac{\hat{y}\ \text{observed} - y'\ \text{calculated}}{\sqrt{V(\hat{y}\ \text{obs.} - y'\ \text{calc.})}}; \quad V(\hat{y}\ \text{obs.} - y'\ \text{calc.}) = \frac{V(\hat{y}\ \text{obs.} - y'\ \text{calc.})}{n_y}; \quad V(\hat{y}\ \text{obs.} - y'\ \text{cals.}) = Ve\left(1 + \frac{1}{n} + \frac{(\bar{x}\ \text{obs.} - \bar{x})^2}{SS_x}\right)$$

The t subscripts refer to the equation numbers of Table 1.

experimental groups did the t values exceed 2 when experimental collagen values were compared with values predicted from femur length using Equation 2. However in all but Groups 1 and 14 the quantity of skin collagen found was less than predicted. In Group 11 where the greatest deviation was observed the mean predicted value was 9.05 gm. of skin collagen whereas an average of 7.38 gm. per rat was found. The quantity of total body protein expected to be present at the start of the experiment was 72 gm. (19% of body weight (Behnke, Osserman and Welham, 1953)). Thus the skin collagen constituted 12.5% of total body proteins. During the course of the experiment a mean loss of 128 gm. of body weight per rat occurred. It could be expected that an average of 24.3 gm. of protein was lost per rat. In conformity with this prediction we have observed a loss of 22.6 gm. of protein per rat in which a mean body weight loss of 126 gm. took place following the administration of cortisone to a group of animals with similar starting weights. A loss of 3.03 gm. of skin collagen would be expected if the loss of collagen was proportional to the loss of protein elsewhere. The difference between observed and calculated was 1.67 gm. It must be concluded that the collagen of skin is at least relatively stable in the face of large losses in body protein induced by the experimental procedures under discussion. The stability of collagen in the skin was anticipated from previously discussed evidence (Sobel, Zutrauen and Marmorston, 1953).

Effect of Treatment upon the Collagen Content of Femurs.—Evidence for the stability of collagen is more definite from inspection of the results of analysis of the femurs of the experimental animals. In two instances the t values exceed 2, but in a positive direction. The various groups showed collagen contents above and below that predicted from Equation 4.

Effect of Treatment upon the Hexosamine Content of Skin.—All experimental groups on stock diet receiving doses of cortisone acetate of 20 mg. per kilo body weight or above had significantly less hexosamine content in their skin than was predicted from Equation 3. This loss did not take place in rats receiving synthetic diets containing 8% and 60% casein. In Group 11 subjected to semi-starvation a significant loss of hexosamine occurred also. It is, of course, well recognized that the intake of food drops markedly in rats treated with cortisone. Rats maintained in the cold room do not respond with a loss of skin hexosamine.

Effect of Treatment upon the Hexosamine Content of the Femurs.—The loss of hexosamine observed with skin under the influence of cortisone is even more strikingly seen with the femurs. In all rats receiving doses of cortisone acetate of 20 mg. per kilo body weight or above, the hexosamine content was significantly less than that predicted from Equation 5. However the groups receiving the synthetic diets lost less hexosamine than the others.

One of the semi-starvation groups showed large losses as did the male rats receiving thyroxine. As with the skin, the femurs of animals exposed to cold did not show a loss of hexosamine.

Effect of Treatment upon the Mineral Content of the Femurs.—Although several groups showed large significant losses of mineral content from their femurs, the results were not consistent and are difficult to interpret.

DISCUSSION

The results of this investigation do not help materially to explain the pathogenesis of the skin and bone lesions of Cushing's disease. Osteoporosis in man has a marked predilection for the vertebrae and pelvis and seldom involves the extremities (Albright and Reifenstein, 1948). Consequently the femur may not be a satisfactory bone to study the effect of the administration of large doses of cortisone. Furthermore, Follis (1951) has reported that in the growing rat cortisone causes a zone of increased density to appear at the ends of the long bones due to a decrease in the normal osteolytic sequences. This is in contrast to pathological observations in man. In several experimental groups large losses of bone minerals were observed, but this finding was not constant. In addition several groups of femurs were investigated radiologically before analysis and there was no evidence of osteoporosis.

The experimental findings indicate that the collagen in the femurs and probably the skin of the rat is at least relatively stable in the face of very large losses in body weight. Significant losses in hexosamine content can be induced by the administration of large doses of cortisone. Hexosamine is a constituent of mucopolysaccharides including hyaluronic acid which is the chief mucopolysaccharide of the ground substance of skin and bone. The decrease in hexosamine content of skin and femurs is consistent with the suggestion that a decrease in the content of hyaluronic acid or other mucopolysaccharides in these organs precedes the events which lead to the development of osteoporosis and striae in Cushing's disease.

Finally it should be pointed out that the ratio of hexosamine to collagen decreases with increase in size of the rat (Sobel, Zutrauen and Marmorston, 1953). It was suggested that this might be a fundamental aspect of the aging process, since the decrease in the ground substance along with an absolute or relative increase in collagen might interfere with the passage of material into and out of cells. Since the hexosamine content decreases while the collagen content does not, the hexosamine to collagen ratio decreases under the influence of cortisone.

SUMMARY

The effect of the administration of cortisone acetate upon the collagen and hexosamine content of the skin and femurs of one year old rats was

investigated. Collagen in these organs appears to be relatively stable in the face of very large losses in weight induced by this substance, as well as by administration of thyroxine, semi-starvation or exposure to cold. The hexosamine content however appears to be diminished, especially in the femurs following cortisone administration.

Acknowledgments

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THE SECRETION OF AN ANTIDIURETIC SUBSTANCE INTO THE CIRCULATION OF ADRENALECTOMIZED AND HYPOPHYSECTOMIZED RATS EXPOSED TO NOXIOUS STIMULI[1,2]

I. ARTHUR MIRSKY, MARVIN STEIN[3] AND GERHARD PAULISCH

With the technical assistance of ROBERT JINKS

Department of Clinical Science, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

THE exposure of normal rats to a variety of noxious stimuli (pain, noise, a strange environment, and the intraperitoneal injection of histamine) results in a rapid, marked increase in the antidiuretic activity of the plasma (Mirsky, Stein and Paulisch, 1953). Although the precise nature of the substance (or substances) (ADS) responsible for the augmented antidiuretic activity was not established, its resemblance to the antidiuretic hormone (ADH) extractable from the neurohypophysis and the speed with which it is released into the circulation suggested that ADS and ADH may be identical. It was proposed that common to the specific reactions induced by the various noxious stimuli is an activation of the hypothalamus with a resultant release of the antidiuretic hormone into the circulation.

It is generally assumed that the secretion of the antidiuretic hormone is dependent upon the integrity of the supraoptic and paraventricular nuclei, the supraoptico-hypophyseal tract and the neurohypophysis (Fisher, Ingram and Ranson, 1938; Verney, 1948; Harris, 1951; Pickford, 1952). In addition, the secretion of the antidiuretic hormone appears to be influenced by the adrenal gland since adrenalectomy produces an increase in the antidiuretic activity of the serum (Birnie, Jenkins, Eversole and Gaunt, 1949) and plasma (Mirsky, Paulisch and Stein, 1953) of the rat.

Although hypophysectomy results in a diminution of the antidiuretic activity of the serum (Birnie, Eversole, Boss, Osborn and Gaunt, 1950)

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[2] Aided in part by a grant from the Eli Lilly Company.

[3] U. S. Public Health Service Research Fellow of the National Institute of Mental Health.

and the plasma of rats (Mirsky, Paulisch and Stein, 1953), adrenalectomy is still effective in producing an increase in the activity of the plasma of hypophysectomized rats (Mirsky, Paulisch and Stein, 1953). Accordingly, it was proposed that an insufficiency of the corticosteroids produced by the zona glomerulosa acts as a non-specific noxious systemic stimulus to activate the release of antidiuretic hormone from some site other than the neurohypophysis. To test this hypothesis, it became pertinent to determine the effect of exposure to noxious stimuli on the antidiuretic activity of the plasma of adrenalectomized and hypophysectomized rats.

METHOD

Male albino rats of the Carworth strain, weighing from 140 to 250 gm. were used. The procedures for producing the noxious stimuli, the method for the assay of the antidiuretic activity of pooled plasma from 4 to 8 rats, and the method for the measurement of the diuretic response to a water load were the same as those employed in a previous study (Mirsky, Stein and Paulisch, 1953). Pitressin[4] was used as the standard in the bioassays and therefore the antidiuretic activity of the plasma is expressed as equivalents of mU Pitressin per 100 ml.

The techniques employed in the preparation and maintenance of the adrenalectomized and the hypophysectomized rats were identical with those reported in the previous study (Mirsky, Paulisch and Stein, 1953).

RESULTS

Response of Adrenalectomized Rats to Painful Stimuli

Three groups of adrenalectomized rats were exposed to a two minute period of repetitive mild electric shocks applied to the feet through a grid floor in individual metabolic cages. One group of 25 rats was studied 18 hours after adrenalectomy during which interval the rats were permitted free access to food and tap water. A second group of 32 rats was also studied 18 hour after adrenalectomy but each rat was given 2 mg. cortisone intraperitoneally immediately after the operation, 1 mg. subcutaneously in 8 hours and 1 mgm. intraperitoneally in 16 hours. The third group consisted of 25 rats which were given a daily subcutaneous injection of 2 mg. cortisone for seven days. The animals were decapitated and bled within 30 seconds after exposure to the painful stimuli.

It is apparent from Figure 1 that exposure of adrenalectomized rats to the painful stimuli resulted in an increase in the concentration of the plasma ADS. Although the activity of the plasma of the untreated 18 hour adrenalectomized rats was significantly greater than that of normal rats ($P<0.001$), exposure to painful stimuli resulted in a further statistically significant increase ($P=0.02$–0.05). The plasma ADS concentration of the untreated 18 hour adrenalectomized rats was 49.0 ± 8.3 mU (9 assays)

[4] We are indebted to Dr. D. A. McGinty of the Parke Davis Company for a supply of Pitressin which assayed to 40 U.S.P. Units per mg.

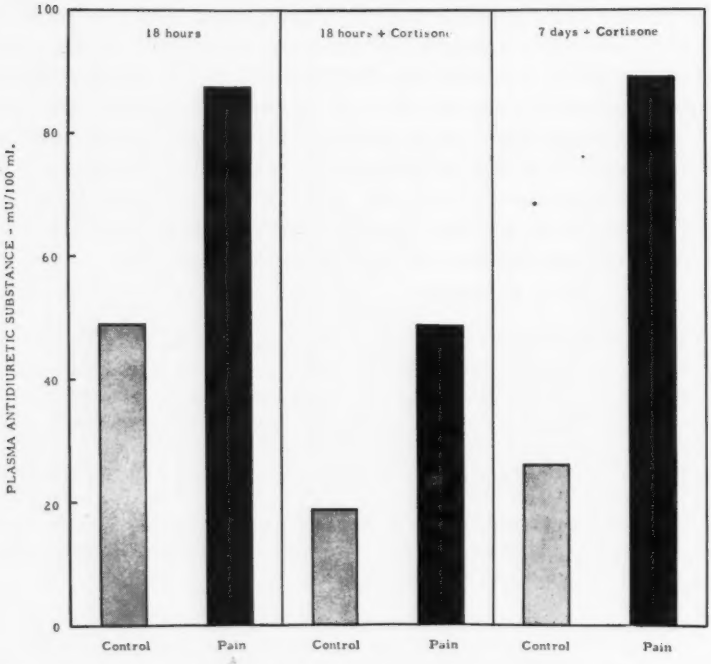

FIG. 1. The antidiuretic activity of the plasma of cortisone-treated and untreated adrenalectomized rats exposed to painful stimuli (see text).

while that of similar animals was 87.4 ± 13.6 mU (4 assays) in 30 seconds after exposure to the painful stimuli. Exposure of the cortisone treated 18 hour adrenalectomized rats to the noxious stimuli resulted also in a statistically significant increase ($P = 0.001$–0.01) from 19.2 ± 0.9 mU to 42.2 ± 6.1 mU per 100 ml. (5 assays). Much more striking was the effect of exposure to painful stimuli on the plasma ADS of the cortisone treated 7-day adrenalectomized rats in that there was an increase from 26.2 ± 3.5 to 89.2 ± 17.9 mU per 100 ml. (5 assays) ($P = 0.001$–0.01).

Response of Hypophysectomized Rats to Painful Stimuli and to Noise

Exposure for two minutes to repetitive mild electric shocks to the feet resulted in a marked increase in the concentration of ADS in the plasma of from 5 to 10 day hypophysectomized rats (Figure 2). Whereas the unexposed animals had a titer of 10.9 ± 2.0 mU per 100 ml., the ADS concentration of the plasma of 38 exposed animals was 127.1 ± 20.1 mU per 100 ml. (5 assays) within 30 seconds after the exposure ($P < 0.001$).

Exposure of hypophysectomized rats for 5 minutes to the noise produced

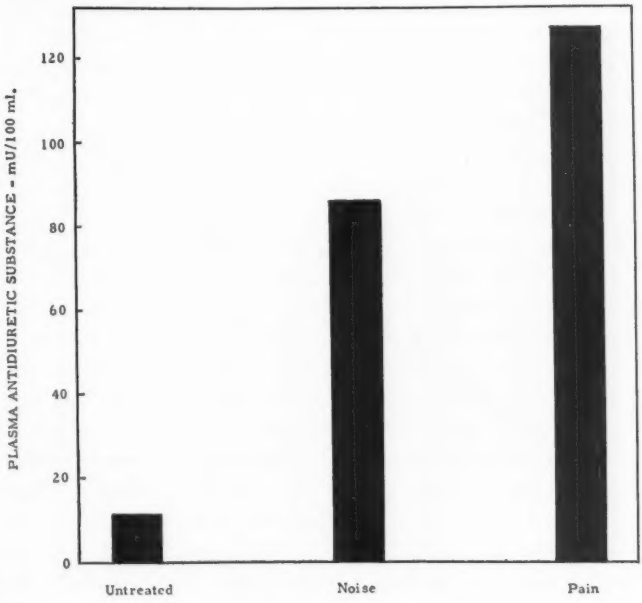

FIG. 2. The antidiuretic activity of the plasma of hypophysectomized rats exposed to noise and to painful stimuli (see text).

by a Federal Siren resulted in a marked increase in antidiuretic activity of the plasma from 10.9 ± 2.0 mU to 65.8 ± 13.6 mU (4 assays). The difference between the non-exposed and exposed hypophysectomized rats was statistically highly significant ($P = 0.001$–0.01).

In order to demonstrate a relationship between the effect on the antidiuretic activity of the plasma and the excretion of urine by hypophysectomized rats, the effect of painful stimuli on the diuresis produced by a water load was determined. Although hypophysectomized rats have a markedly diminished diuretic response to a water load, a partial restitution occurs with the administration of adrenal cortical hormones (Boss, Birnie and Gaunt, 1950). Accordingly, 6 days after hypophysectomy, 10 rats were each given 2 mg. cortisone intraperitoneally. Two hours after the injection, each rat was given a gavage of 5 ml. 0.2% NaCl per 100 gm. of body weight and one hour later, the gavage was repeated and the volume of urine excreted recorded every 20 minutes thereafter. At the end of the first twenty minutes after the second gavage, the animals were exposed to repetitive mild electric shocks to the feet for two minutes and the effect on the urine flow noted. Three days later, the animals were again given cortisone and diuresis was induced as before but the animals were not

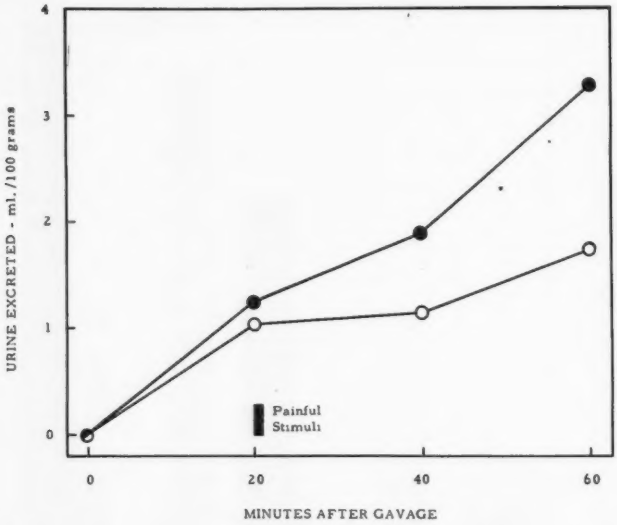

FIG. 3. The antidiuretic response of hypophysectomized rats exposed to painful stimuli. Cortisone was administered three hours before. Five ml. of 0.2% NaCl per 100 gm. body weight were given at one hour before and again at 0 time. At the time designated by the block, the rats were exposed for two minutes to repetitive mild electric shocks to the feet (○——○). Subsequently the same rats were tested without exposure to painful stimuli (●——●) (see text).

exposed to any noxious stimuli. It is apparent from Figure 3 that exposure to the painful stimuli results in a marked inhibition of the diuresis induced by the water load. Prior to the exposure, there was no significant difference in the volume of urine excreted ($P <= 0.2$–0.3) but after the exposure the differences were statistically highly significant at both the 40 and 60 minute intervals ($P = 0.01$–0.2 and $P < 0.001$).

DISCUSSION

The data reported herein reveal that neither the hypophysis nor the adrenal gland is essential for the increase in the antidiuretic activity of the plasma which occurs in rats exposed to noxious stimuli. Likewise, the hypophysis is not essential to the antidiuretic response of adrenalectomized rats (Mirsky, Paulisch and Stein, 1953). Consequently, the major source of the antidiuretic activity of the plasma of the rat must be some site other than the neurohypophysis.

Two principal hypotheses have been proposed regarding the site of origin of the antidiuretic hormone. The commonly accepted hypothesis postulates that the antidiuretic hormone, vasopressin and oxytocin are secreted in the neurohypophysis and that such secretion is dependent upon the integrity of

the nerve supply from the supraoptic and paraventricular nuclei (Fisher, Ingram and Ranson, 1938; Verney, 1948; Harris, 1951a; Pickford, 1952). The second hypothesis attributes the secretion of the hormones of the neurohypophysis to the cells of the supraoptic and paraventricular nuclei, the neurohypophysis acting as the site of storage rather than that of secretion (Scharrer, 1933; Gaupp and Scharrer, 1935; Scharrer and Scharrer, 1945; Bargmann, 1949; Stutinsky, 1951; Hild, 1951a; Bargmann and Scharrer, 1951; Ortman, 1951; Scharrer and Wittenstein, 1952; Hild and Zetler, 1953; Leveque and Scharrer, 1953; Rothballer, 1953). Smith's observations (1951) suggest that the mammillo-infundibular nuclei are also involved in the secretory process. Bodian (1951) extended this concept and proposed that the entire supraoptico-hypophyseal system participates in the secretion of the neurohypophyseal hormones.

In support of the hypothesis that the supraoptic and paraventricular nuclei are the site of secretion of the neurohypophyseal hormones is the demonstration that whereas extracts of the rest of the nervous system are devoid of any antidiuretic activity, extracts of the hypothalamus of various species contain significant amounts of this hormone (Abel, 1923; 1924; Sato, 1928; Trendelenberg, 1929; Melville and Hare, 1945; Kovacs and Bachrach, 1951; Hild and Zetler, 1951a, 1951b, 1952a, 1952b, 1953). The supraoptic nuclei are particularly rich in ADH, containing 15 to 25% as much as does the neurohypophysis (Melville and Hare, 1945). Likewise, extracts with vasopressin and oxytocic activities have been prepared from the hypothalamic nuclei of many species (Hild and Zetler, 1951a, 1951b, 1952a, 1952b, 1953). Hild and Zetler (1953) demonstrated that extracts of the supraoptic nuclei are more potent in antidiuretic and vasopressin activities while extracts of the paraventricular nuclei are more potent in oxytocic activity. Even after removal of the neurohypophysis (Melville and Hare, 1945), or section of the stalk (Hild and Zetler, 1953), the hypothalamus contains appreciable quantities of antidiuretic hormone.

Most of the evidence in favor of the hypothalamic origin of the hormones of the neurohypophysis is based on the demonstration of specifically staining "neurosecretory granules" in the supraoptic, paraventricular and mammillo-infundibular nuclei, the supraoptico-hypophyseal tract and the neurohypophysis. Bargmann and Scharrer (1951), Leveque and Scharrer (1953) and Hild and Zetler (1953) have reviewed some of the evidence which indicates that the "neurosecretory granules" are concerned with the secretion, transport and discharge of the antidiuretic hormone. Water deprivation results in an increase in the antidiuretic activity of the urine (Gilman and Goodman, 1937) and plasma (Mirsky, Stein and Paulisch, 1953), a decrease in the antidiuretic activity of hypothalamic and neurohypophyseal extracts (Kovacs and Bachrach, 1951; Hild and Zetler, 1953) and a disappearance of the "neurosecretory granules" from the neuro-

hypophysis, the supraoptico-hypophyseal tract, and the supraoptic and paraventricular nuclei (Ortmann, 1951; Hild, 1951; Hild and Zetler, 1953; Leveque and Scharrer, 1953). Likewise, the administration of a hypertonic solution of sodium chloride produces an antidiuretic response (Verney, 1947), a decrease in the antidiuretic activity of hypothalamic and neurohypophyseal extracts (Kovacs and Bachrach, 1951) and a depletion of the "neurosecretory granules" of the neurohypophysis (Ortmann, 1951; Hild, 1951; Leveque and Scharrer, 1953). The administration of water to dehydrated rats results in restitution of the "neurosecretory granules" (Ortman, 1951; Leveque and Scharrer, 1953; Hild and Zetler, 1953) and in the antidiuretic activity of extracts of the hypothalamus and neurohypophysis (Hild and Zetler, 1953).

Particularly pertinent to the present study are the observations that exposure to noxious stimuli results in an inhibition of the diuresis induced by a water load in the dog (Verney, 1947), the rat (Mirsky, Stein and Paulisch, 1953) and man (Kelsall, 1949), an increase in the antidiuretic activity of the plasma of the rat (Mirsky, Stein and Paulisch, 1953) and man (Mirsky and Stein, 1953), a drecrease in the antidiuretic activity of extracts of the neurohypophysis and hypothalamus of the rat (Kovacs and Bachrach, 1951), and a loss of the "neurosecretory granules" from the neurohypophysis and hypothalamus of the rat (Rothballer, 1953). Accordingly, the increased antidiuretic activity of the plasma of the hypophysectomized rat after exposure to noxious stimuli may be due to the discharge of ADH directly from the hypothalamus rather than from the neurohypophysis. Such a conclusion is in agreement with that of Keller (1942) who demonstrated that the neurohypophysis is not essential for the maintenance of a normal water balance in cats and dogs.

The aforementioned is not in accord with the conclusion of O'Conner and Verney (1942) that the antidiuretic response to a noxious stimulus is dependent upon the presence of an intact pars nervosa. These investigators measured the decrease in the diuresis induced in dogs by the administration of water by stomach tube as an index of the antidiuretic response. After removal of the posterior hypophysis, their dogs showed a slight decrease in the diuretic response to hydration and a relatively insignificant antidiuretic response on subsequent exposure to painful stimuli. In the present study, completely hypophysectomized rats were used. Such animals may show no significant disturbance in the daily spontaneous intake and output of water but develop a marked decrease in the rate of urine excretion after the admistration of water by stomach tube (Boss, Birnie and Gaunt, 1950; Hofmann, 1952). For example, during the first hour after the administration by stomach tube of 5 ml. of 0.2% NaCl per 100 gm. body weight, the mean excretion ($\pm$S.E.) of a group of normal rats was 2.32 ± 0.09 ml.

per 100 gm. body weight, while that of a group of similarly treated completely hypophysectomized rats was only 0.27 ± 0.05 ml. per 100 gm. (Paulisch and Mirsky, 1953). Since these hypophysectomized rats showed no significant disturbance in daily water exchange, it is probable that the intubation per se is in some way responsible for the delayed excretion. Exposure of such animals to noxious stimuli cannot be expected to produce any further significant diminution in excretion. When, however, cortisone is administered prior to the intubation, a partial restitution of the diuresis occurs. Under such circumstances, exposure to painful stimuli results in a significant decrease in the diuresis. Consequently, it is possible that the diminished antidiuretic response to noxious stimuli that is noted in the dog after removal of the posterior hypophysis is due to the manner in which the water was administered. It is possible also that the discrepancy between the data reported herein and those of O'Conner and Verney (1942) is due principally to species differences. Hare (1939), however, demonstrated that cats and dogs with diabetes insipidus can develop an antidiuretic response to noxious stimuli but he attributed the response to a decrease in glomerular filtration.

The same noxious stimuli which elicit an antidiuretic response and an increase in the antidiuretic activity of the plasma in normal, adrenalectomized and hypophysectomized rats can initiate also the sequence of events described by Selye (1936) as the "alarm reaction." A decrease in the rate of urine excretion and in the diuretic response to the ingestion of a water load is noted only during the early "shock" phase of the "alarm reaction." Thereafter, with the appearance of other manifestations of an increased adrenal cortical activity, the output of urine may become normal or even excessive. In the absence of the adrenals, the stimuli which initiate the "alarm reaction" may result in a more intense oliguria than when the adrenals are intact (Howlett and Browne, 1937; Karady, Browne and Seyle, 1938; Browne, Karady and Selye, 1939; Howlett and Browne, 1940; Seyle, 1951). The oliguria of the "alarm reaction," therefore, is independent of and precedes the activation of the adrenal cortex. Since an increase in the reabsorption of water by the renal tubules is a major factor in the oliguria of the rat with adrenal insufficiency (Gaunt, Birnie and Eversole, 1949), it may be assumed that the oliguria is due in part at least to the release of ADH into the circulation. In accord are the observations that exposure to noxious stimuli or adrenalectomy results in a decrease in the antidiuretic activity of hypothalamic and neurohypophyseal extracts (Kovacs and Bachrach, 1951) and an increase in the antidiuretic activity of the plasma of the rat. The rapidity with which the "neurosecretory" material of the hypothalmus is mobilized and enters the circulation (Rothballer, 1953) and the rapidity with which the antidiuretic activity

of the plasma is increased in the normal or adrenalectomized rat favors the hypothesis that the secretion of ADH precedes the activation of the adrenal cortex.

Since the discharge of ADH appears to precede the activation of the adrenal cortex and since the release of adrenocorticotrophic hormone (ACTH) is essential to the latter, it is pertinent to consider the possibility that the discharge of ADH by the hypothalamus and the discharge of ACTH by the adenohypophysis are related. Both events occur in response to noxious stimuli or to adrenalectomy. Both events occur at a very rapid rate: the ACTH content of the blood "is significantly increased within two minutes after subjecting animals to stressful stimuli" (Snydor and Sayers, 1953), while the antidiuretic activity of the plasma is likewise maximal by two minutes after the onset of such stimuli (Mirsky, Stein and Paulisch, 1953). Finally, a relationship between the discharge of ADH into the circulation and the activation of the adenohypophysis is suggested by the decrease in adrenal ascorbic acid which ensues after the administration of vasopressin to rats (Nagarada and Gaunt, 1951; Gray and Munson, 1951).

Activation of the hypothalamus is a common response to the different specific reactions induced by a variety of noxious stimuli. There is much evidence that such activation is essential not only to the secretion of ADH but also to the discharge of the hormones of the adenohypophysis (Markee, Sawyer and Hollinshead, 1948; Harris, 1948; Hume, 1949; Hume, and Wittenstein, 1950; deGroot and Harris, 1950; Harris, 1952; Hume, 1952; Fortier, 1952; Porter, 1952; French, Longmire, Porter and Movius, 1953; McCann, 1953). The evidence also favors the hypothesis that the activation of the adenohypophysis by the hypothalamus is dependent upon the secretion of some neurohormone (or neurohormones) which is transported from the hypothalamus to the adenohypophysis by the hypophyseal portal blood supply (Harris, 1948; Green and Harris, 1949). It is possible also that in response to noxious stimuli, the neurohormone is released into both the hypophyseal portal vessels and the general circulation (Hume and Wittenstein, 1950; Rothballer, 1953).

The nature of the hypothalamic neurohormone is unknown. Markee, Sawyer and Hollinshead (1948) suggest that both a cholinergic agent and an adrenergic agent are involved in the secretion of gonadotrophic hormones by the adenohypophysis. Harris, Jacobsohn and Kahlson (1952) suggest that the neurohormone responsible for the discharge of ACTH may be a histamine-like agent but Guillemin and Fortier (1953) could not support this hypothesis since the release of ACTH was not prevented by preliminary treatment with antihistaminics. Nor-epinephrine (Vogt, 1952) and substance P (Pernow, 1951) have also been proposed as the hypothalamic neurohormone. That the antidiuretic hormone (vasopressin)

and/or oxytocin may play a role in the discharge of ACTH and other trophic hormones, is suggested by the considerations given above and by the demonstration that the "neurosecretory" material of the stalk and median eminence is released rapidly into the hypophyseal portal vessels after exposure of the rat to painful stimuli (Rothballer, 1953).

Stimuli which result in a discharge of ADH into the circulation may induce a concomitant discharge of oxytocin (Cross, 1950; Anderson, 1951; Harris, 1951) and a reduction in the oxytocic activity of hypothalamic and neurohypophyseal extracts (Hild and Zetler, 1953). The quantity of hormones discharged may depend upon the type of stimulus. Thus, Cross (1950) noted a slight antidiuretic and a marked oxytocic response (milk-ejection) in lactating rabbits suddenly exposed to their suckling young. Andersson (1951) demonstrated that the injection of a hypertonic sodium chloride solution which results in a marked antidiuretic response (Verney, 1947), produces also a discharge of oxytocin. If the antidiuretic and vasopression activities are due to a single agent, then there are at least two distinct polypeptides, vasopressin and oxytocin (duVignaud, Ressler, Swan, Roberts, Katsoyannis and Gordon, 1953) which may be secreted by the hypothalamus and discharged from the hypothalamic-neurohypophyseal system into the general circulation and into the hypophyseal portal vessels after exposure to noxious stimuli. It is quite possible that one of these agents acts specifically on the acidophilic and the other on the basophilic cells of the adenohypophysis.

Besides their action on peripheral systems and their postulated role in the activation of the adenohypophysis, vasopressin and oxytocin may activate neuronal centers in the hypothalamus. Cushing (1931a, 1931b) demonstrated that the injection of "pituitrin" into the third ventricle produced peripheral effects which are attributable to a massive parasympathetic discharge. The intravenous injection of "pituitrin" was ineffective in this regard, presumably due to the blood-brain barrier. Subsequently, Light and Bysshe (1933) established that the parasympathetic discharge was due to the action of the vasopressin in the "pituitrin." It may be postulated, therefore, that vasopressin, like other neurohormones, acts also at or near the site of its production to stimulate the parasympathetic centers in the anterior hypothalamus. Accordingly, the response to a noxious stimulus is the resultant of the interaction of neuronal and hormonal influences on both central and peripheral mechanisms.

SUMMARY

Exposure of adrenalectomized and hypophysectomized rats to noxious stimuli results in a marked increase in the antidiuretic activity of the plasma. Exposure of cortisone-treated hydrated, hypophysectomized rats to such stimuli results in an antidiuresis. Consequently, neither the adre-

nal gland nor the hypophysis is the source of the factor responsible for the increased antidiuretic activity of the plasma of animals exposed to noxious stimuli.

Various considerations support the hypothesis that the increased antidiuretic activity of the plasma of the rat after exposure to noxious stimuli is due to the antidiuretic hormone (vasopressin) which is secreted by the various hypothalamic nuclei and discharged into the circulation. It is proposed that the antidiuretic hormone (vasopressin) and oxytocin may serve as the hypothalamic "neurohormones" responsible for activation of the adenohypophysis in response to noxious stimuli.

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ANTERIOR PITUITARY GROWTH HORMONE ADMINISTRATION IN B VITAMIN DEFICIENCIES IN THE RAT·

J. L. BEARE, J. R. BEATON AND E. W. McHENRY

Department of Public Health Nutrition, University of Toronto, Toronto, Canada

IT HAS been previously reported from this laboratory that administration of anterior pituitary growth hormone to pyridoxine-deficient rats results in an accentuation of the deficiency syndrome (Beare, Beaton, Smith and McHenry, 1953). Further, the hormone was unable to exert its anabolic properties in rats not provided with pyridoxine but provided with the other essential B vitamins. It was stated that only one vitamin had been studied and that the specificity of effects was not known. The experiments to be reported represent similar investigations employing rats deficient in other members of the vitamin B complex.

METHODS

In both experiments, albino rats of the Wistar strain and of both sexes were employed. All animals were housed in individual screen-bottomed cages during the initial period of experimental feeding and vitamin deprivation. Food and water were provided *ad libitum*. When vitamin deficiencies were apparent, the respective groups were rebalanced in average body weight and transferred to metabolism cages (with 4 or 5 animals in each cage) for a further 10 days of experimental feeding. During the last 7 days, 1 group on each dietary regimen received single daily subcutaneous injections of 1 mg. anterior pituitary growth hormone preparation[1] in 1 cc. saline. Injections of saline of the same volume were given to the remaining groups. During this 7-day injection period, urine and feces were collected daily for each group and separately pooled for 3-day intervals. Toluene was employed as the urine preservative. At the termination of experimental feeding, the animals were fasted for 20 hours and anesthetized by the intraperitoneal administration of 2% nembutal in saline. Blood was removed from the exposed heart, pooled for each group and heparinized. Carcasses were pooled for each group, frozen, passed through a power grinder and homogenized. The analytical procedures were those previously described (Beare, Beaton, Smith and McHenry, 1953).

EXPERIMENTAL

I. *Riboflavin and pantothenic acid deficiencies.*—Forty-eight rats were divided into 6 groups equal with respect to initial average body weight (160 gm.), sex distribution and number. All animals were provided with

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[1] Armour Laboratories 22KRI.

TABLE 1. AVERAGE BODY WEIGHT CHANGES, FOOD CONSUMPTIONS AND NITROGEN RETENTION OF EXPERIMENTAL ANIMALS

Experiment	Group	Average body weight change		Average daily food consumption		Average nitrogen retention
		Before injections	During injections	Before injections	During injections	
		gm.	gm.	gm.	gm.	gm./rat /day
I	control	125	−5	18.6	11.1	.13
	control G.H.[1]	125	23	18.6	11.4	.20
	-riboflavin	15	6	14.1	10.6	.21
	-riboflavin G.H.	15	6	14.1	10.9	.27
	-pantothenate	65	4	15.2	12.3	.22
	-pantothenate G.H.	65	4	15.2	11.3	.22
II	control	87	14	13.5	9.9	0.18
	control G.H.	87	19	13.5	9.5	0.17
	-thiamine	28	−5	9.7	3.1	0.02
	-thamine G.H.	28	6	9.7	4.1	0.08

[1] G.H. indicates groups injected with growth hormone.

the 20% casein, 20% corn oil basal diet previously described (Beare, Beaton, Smith and McHenry, 1953) with the following changes in vitaminized casein: 100 mg. of pyridoxine hydrochloride was added and riboflavin and calcium pantothenate were omitted. The following vitamin supplements were added to the food of each rat per day as indicated: riboflavin 50 μg.; calcium pantothenate 200 μg. Two groups received both vitamin supplements and served as controls; two groups received calcium pantothenate but no riboflavin; two groups received riboflavin but no calcium pantothenate. Following 49 days of experimental feeding and treatment as described under Methods, the animals were fasted and sacrificed. The results of analyses are shown in Tables 1, 2 and 3.

TABLE 2. BLOOD ANALYSES OF EXPERIMENTAL ANIMALS

Experiment	Group	Blood			
		Packed cell volume	Hemoglobin	Urea	Plasma glutamic acid
		%	gm. %	mg. %	mg. %
I	control	46	13.7	28	3.0
	control G.H.[1]	46	12.7	26	5.9
	-riboflavin	44	13.3	43	3.0
	-riboflavin G.H.	46	13.5	35	4.6
	-pantothenate	43	13.5	28	2.8
	-pantothenate G.H.	47	13.1	22	4.2
II	control	41	13.5	28	2.2
	control G.H.	41	12.5	25	2.7
	-thiamine	45	13.3	50	3.3
	-thamine G.H.	46	12.3	28	2.9

[1] G.H. indicates groups injected with growth hormone.

TABLE 3. CARCASS COMPOSITION OF EXPERIMENTAL ANIMALS

Experiment	Group	Total crude fatty acids	Protein	Moisture
		%	%	%
I	control	17.8	21.8	61.7
	control G.H.[1]	18.3	21.6	62.9
	-riboflavin	9.5	23.1	66.7
	-riboflavin G.H.	6.9	23.1	69.2
	-pantothenate	13.9	22.6	64.5
	-pantothenate G.H.	11.2	21.8	65.8
II	control	15.8	20.5	
	control G.H.	15.5	20.7	
	-thamine	4.7	22.5	
	-thiamine G.H.	4.3	21.6	

[1] G.H. indicates groups injected with growth hormone.

II. *Thiamine deficiency.*—Since rats not provided with thiamine would not survive a 49-day experimental period as in experiment I, a shorter term experiment was designed to investigate thiamine deprivation. Forty rats were divided into 4 groups comparable with respect to initial average body weight (105 g.), sex distribution and number. The basal diet was the 20% casein, 20% corn oil diet with the following change in the vitaminized casein: 100 mg. of pyridoxine hydrochloride replaced thiamine chloride. Two groups received 50 μg. of thiamine chloride per rat per day in their food; two groups received no thiamine. Following 29 days of experimental treatment as described under Methods, the animals were fasted and sacrificed. The analytical results are set down in Tables 1, 2 and 3.

RESULTS AND DISCUSSION

All vitamin-deficient groups exhibited typical inanition and decreased body weight gains. Administration of growth hormone, although causing slight to marked gains in average body weights of control animals, failed to effect this gain in those deprived of riboflavin or pantothenate. Based on body weight changes, Lotspeich (1950) has previously suggested that pantothenic acid-deficient rats are unable to respond anabolically to growth hormone administration. In contrast, growth hormone appeared to cause a body weight gain in rats deprived of thiamine. In confirmation of earlier observations (Beare, Beaton, Smith and McHenry, 1953) it would appear that the body weight gain in growth hormone-injected controls was not a consequence of a marked increase in food consumption during the injection period. From the results of blood analyses, certain observations should be noted. Neither the vitamin deficiencies nor growth hormone administration had any significant effect upon packed cell volumes nor hemoglobin levels. Deficiencies of thiamine and riboflavin, but not of pantothenic acid, caused marked elevations in fasting blood urea such as have been observed in rats deprived of pyridoxine (Hawkins, MacFarland and

McHenry, 1946). Growth hormone appeared to depress fasting blood urea levels in these 3 vitamin deficiencies, in contrast with the further elevation noted in pyridoxine-deficient rats given growth hormone. Plasma glutamic acid levels were normal in thiamine, riboflavin and pantothenic acid-deprived rats as has been observed in pyridoxine-deficient rats in this laboratory. Administration of anterior pituitary growth hormone appeared to cause increases in fasting plasma glutamic acid in all groups except the thiamine-deprived group though an interpretation of this phenomenon is not available at present.

Deficiencies of thiamine, riboflavin and of pantothenic acid all resulted in a marked lowering of per cent total crude fatty acids in the carcass accompanied by small relative increases in per cent protein and per cent moisture. Thus the carcass compositions in these B vitamin deficiencies are of the same pattern as in pyridoxine deficiency (Beare, Beaton and McHenry, 1953). Growth hormone administration appeared to accentuate these abnormalities in riboflavin and pantothenic acid-deprived rats as was previously shown for rats deprived of pyridoxine. No clear-cut accentuation was observed in thiamine-deficient animals. It is evident from the experimental data that the increase in body weight of control animals as a consequence of growth hormone administration was approximately equally distributed between total crude fatty acids, moisture and protein.

As would be anticipated from body weight changes, growth hormone administration caused an increased nitrogen retention in the control animals of Experiment 1 but not of Experiment II. In the absence of exogenous growth hormone, rats deprived of thiamine had a lower nitrogen retention than did control animals. In contrast, riboflavin-deprived and pantothenic acid-deprived animals had a higher level of nitrogen retention than did the controls in Experiment 1. This observation is probably a consequence of the plateauing of body weight in the control rats following the extended feeding period whereas the riboflavin and pantothenic acid-deprived rats actually gained slightly in body weight during the last week of experimental feeding. A slight increase in nitrogen retention following growth hormone administration was noted in riboflavin-deprived animals. It should be noted that pyridoxine-deficient rats were unable to respond to growth hormone by increased nitrogen retention (Beare, Beaton, Smith and McHenry, 1953).

Deficiencies in four of the B vitamins have been studied in the rat in this laboratory to determine their effects on the response to growth hormone. Although thiamine deficiency did not appear to interfere with the anabolic response to growth hormone, an interference was produced by riboflavin, pantothenic acid and pyridoxine deficiencies. It is realized that these deficiencies were produced by complete dietary restriction of these vitamins, however it is felt that in studies on the effects of growth hor-

mone administration, careful consideration must be given to the constituents of the diet as well as to the amount of food consumed. Ershoff and Deuel (1945) have observed that plateaued, vitamin A-deficient rats fail to gain weight when a growth hormone preparation was administered. It may well be that deficiencies of other vitamins will interfere with the anabolic response to growth hormone.

It could be said that all of the results are explicable on the basis of food consumption. If food consumption of control rats had been restricted to the amount eaten by deficient animals, growth hormone might not have had an anabolic effect in the controls. Even the data on thiamine deficiency support this; the growth hormone administration caused a 25% increase in food consumption and this may have made possible the observed anabolic response. However, in the absence of dietary thiamine, rats can respond to growth hormone administration. Apparently, rats deprived of pyridoxine, pantothenic acid or riboflavin cannot respond to the hormone.

SUMMARY

Experiments are described in which anterior pituitary growth hormone was administered to riboflavin-, pantothenic acid- and thiamine-deprived rats. Rats not provided with riboflavin or pantothenic acid were unable to respond anabolically to growth hormone as evidenced by a failure to increase body weight whereas growth hormone caused increased body weight gains and nitrogen retention in thiamine-deprived and control animals. In the absence of dietary thiamine rats can respond anabolically to growth hormone. In all groups, fasting blood urea was depressed by growth hormone administration. The results of these studies indicate that in investigations on the effects of growth hormone administration, careful consideration must be given to dietary constituents as well as to the amount of food consumed.

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EFFECT OF ALTERED THYROIDAL AND GONADAL ACTIVITY ON SIZE OF ENDOCRINE GLANDS AND RESISTANCE TO STRESS IN THE CHICK[1,2]

MARK H. CONNER[3] AND C. S. SHAFFNER

Poultry Department, University of Maryland, College Park, Maryland

ALTHOUGH it is probable that an altered level of any endocrine hormone may affect the level of any of the others, the exact mode and site of these actions are often obscure. For example, it has been suggested that greatly increased production of one anterior pituitary hormone may inhibit production of the others. Selye (1949) described this as the "shift in anterior-lobe hormone production" which follows an increased need for one hypophyseal hormone. Zarrow and Zarrow (1951) attributed the involution of the rat adrenal, which they observed following thiouracil administration, to a decreased ACTH output resulting from excessive TSH secretion. But Hughes (1944) reported that goitrogens have no effect on adrenal weight. Halmi and Bogdanove (1951) showed that the ACTH content of the pituitary of thyroidectomized rats is not significantly different from that of normal controls.

It has also been shown that, in mammals, the activities of the thyroid, adrenals, and gonads are closely related. The nature of these relationships, especially in regard to the role of the adrenal, is not well known in the chick.

In the present study, the nature of some hormonal interactions in the chick were observed. The response of this animal to stress, a factor about which little information is available, was also investigated.

MATERIALS AND METHODS

The experimental animals used in the studies were New Hampshire chicks of both sexes taken at random from the University of Maryland farm stock. The day-old chicks were placed in electrically heated batteries and provided with food and water *ad libitum*.

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[3] Present Address: Poultry Section, Agricultural Research Center, Beltsville, Maryland.

TABLE 1. BODY AND ORGAN WEIGHTS OF BIRDS FED 0.2% THIOURACIL PLUS VARYING AMOUNTS OF THYROPROTEIN[1]

	Thyroprotein, ppm.					
	0.0	11	22	44	66	Control[2]
Body, gm.	213 ± 12	285 ± 7	301 ± 7	301 ± 7	299 ± 6	316 ± 5
Thyroid, mg.	182 ± 19	209 ± 17	153 ± 13	36 ± 10	13 ± 2	21 ± 1
Adrenal, mg.	26 ± 1	26 ± 1	30 ± 1	28 ± 1	29 ± 2	29 ± 1
Testes, mg.	66 ± 7	77 ± 5	83 ± 4	73 ± 4	79 ± 21	68 ± 15
Ovary, mg.	37 ± 2	52 ± 3	53 ± 2	62 ± 3	63 ± 8	62 ± 3
Male comb, mg.	36 ± 6	140 ± 13	176 ± 16	222 ± 31	210 ± 25	243 ± 20
Female comb, mg.	22 ± 1	38 ± 2	46 ± 3	62 ± 4	57 ± 10	67 ± 4
Heart, gm.	1.2 ± 0.0	—	1.8 ± 0.0	2.0 ± 0.1	1.9 ± 0.0	2.0 ± 0.0

[1] Means with their standard errors, calculated from all values in five replicates.
[2] Received no thiouracil or thyroprotein.

At the end of each test, the birds were weighed to the nearest gram and then sacrificed by bleeding at the neck. The carcasses were stored at −14° F. for 1 to 7 days to allow time for dissection. When the birds were dissected, the appropriate organs were carefully removed, trimmed of extraneous tissue, and rapidly weighed. Immediately after weighing, those tissues to be sectioned were placed in Tellyesniczky's fixative. They were then imbedded in paraffin, sectioned at 10 microns with a rotary microtome, and stained with Masson's Trichrome.

To study the effect of altered thyroid activity on thyroid gland size, six groups of 8 to 10 chicks each were selected. These groups were fed a ration to which 0.2% thiouracil plus varying amounts of thyroprotein were added (shown in Table 1). The birds were sacrificed and autopsied at four weeks of age. Observations were made on body weight and weight of thyroid, adrenal, gonads, comb, and heart, respectively. This experiment was repeated five times, from April to July.

To observe the effect of stress on birds at various levels of thyroid activity, nine groups of 8 to 10 chicks each were selected. These groups were fed a ration to which 0.2% thiouracil plus varying amounts of thyroprotein were added (shown in Table 2).

TABLE 2. RESPONSE TO VARYING THYROPROTEIN LEVELS AND STRESS OF BODY AND ORGAN WEIGHTS OF BIRDS FED 0.2% THIOURACIL

	Thyroprotein, ppm.								
	0.0	22	33	44	55	66	77	88	Control[2]
Body, gm.	235 ± 6	313 ± 7	314 ± 8	283 ± 14	285 ± 8	295 ± 7	285 ± 6	303 ± 7	329 ± 9
d[1]	22	12		18		4			13
Thyroid, mg.	170 ± 22	208 ± 20	103 ± 15	36 ± 5	24 ± 6	15 ± 4	8 ± 1	9 ± 2	16 ± 1
d	12	55		0		2			5**
Adnreal, mg.	38 ± 2	43 ± 3	46 ± 2	40 ± 3	40 ± 2	43 ± 2	42 ± 3	43 ± 2	46 ± 2
d	13**	12**		12**		14**			17**
Testes, mg.	70 ± 6	87 ± 6	102 ± 9	87 ± 9	89 ± 10	96 ± 8	97 ± 6	104 ± 9	81 ± 5
d	4	4		14		17			13
Ovary, mg.	44 ± 5	75 ± 5	71 ± 5	64 ± 4	70 ± 3	71 ± 5	70 ± 3	74 ± 4	68 ± 5
d	7	22**		2					6
Male comb, mg.	38 ± 3	187 ± 22	247 ± 26	240 ± 31	322 ± 26	300 ± 43	306 ± 46	393 ± 65	228 ± 28
d	2	11		18		90			15
Female comb, mg.	27 ± 4	72 ± 6	86 ± 10	69 ± 6	64 ± 4	69 ± 5	69 ± 9	68 ± 5	74 ± 8
d	5	26**		7		12			7
Heart, gm.	1.4 ± .1	2.0 ± .1		1.9 ± .1		1.8 ± 0			2.2 ± .1
d	0.2**	0.2		0.1		0.1			0.1
Revs. to exhaust	355	360	465	540	435	375	405	465	405
Range	11–1455	10–1440	30–1590	19–1920	23–1860	19–1650	19–1650	21–1800	13–2070

[1] "d" indicates difference between the above means, secured when the chickens were stressed, and means for comparable treatments without stress shown in Table 1.
** Indicates statistical significance at the 1.0% level.
[2] Received no thiouracil or thyroprotein.

At 17 days of age, all of these birds were run to exhaustion in electrically driven exercise cages, which turned at a speed of 10 revolutions per minute. This stress was then repeated, as follows: Second run, 21 days of age; speed 15 rpm. Third run, 24 days of age; speed 15 rpm. Final run, 27 days of age; speed 15 rpm. The end-point of complete exhaustion was taken as that time when the bird fell to the bottom of the cage and tumbled continuously. Observations were made on length of time necessary to exhaust the bird, body weight, and weight of thyroid, adrenal, gonads, comb, and heart, respectively. Several histological sections were made of the adrenal glands from untreated birds and from birds exposed to stress only, in these groups. This experiment was repeated three times, in August and September.

To test the effect of the sex hormones and gonadotropin on the resistance to fatigue, five comparable groups of 20 birds each were selected and injected as follows: Group 1, control; group 2, testosterone propionate 0.7 mg. per bird; group 3, testosterone propionate 1.4 mg. per bird; group 4, diethylstilbestrol 0.7 mg. per bird; group 5, gonadotropin (Gonadin) 70 units per bird. Testosterone propionate and diethylstilbestrol were dissolved in sesame oil. The injections were divided into seven equal doses which were given every 48 hours during a period beginning three days before the start of exercise and ending 24 hours before autopsy. Half of each group was stressed by the same means and on the same schedule as previously described. Due to the increased resistance to stress shown by the birds treated with sex hormones, the speed of the cages was increased in the third and fourth runs as follows: Third run, 20 rpm.; fourth run, 20 rpm. except for groups 2, 3, 4, 5, which were exercised at 25 rpm.

OBSERVATIONS

The results of the thiouracil-thyroprotein treatment without stress are presented in Table 1. It is apparent that the addition of 11 ppm. thyroprotein to a diet containing thiouracil produced an even greater hypertrophy of the thyroid than did thiouracil alone. When thiouracil plus amounts of thyroprotein increasing from 22 to 66 ppm. were added to the feed, the thyroid size approximated that of the control.

Although body weight was sharply decreased in those groups receiving thiouracil plus little or no thyroprotein, size changes in some organs measured did not necessarily reflect this decrease. The adrenals were only slightly reduced in size by extreme hypothyroidism, yet body size was greatly reduced thereby. However, the combs of both sexes in these hypothyroid groups appeared to be much smaller than might be expected even for birds of this reduced size.

The results obtained with thiouracil and thyroprotein plus the stress of muscular exhaustion are presented in Table 2. By comparing this data with that in Table 1, it may be seen that stress caused a significant increase in adrenal size in all groups receiving thyroprotein as well as in the untreated group. However, stress did not alter the response of the adrenal to thryoprotein.

The gonads of stressed birds of both sexes were somewhat larger than those of the nonstressed birds. This was true for groups receiving thyro-

protein but the only statistically significant difference occurred in the weight of the ovaries of the group which received thyroprotein at the 22 ppm. level. The comb weights paralleled these gonadal responses.

Heart weight of stressed birds was significantly increased in the presence of low thyroxine levels. The increase was most marked in the birds receiving thiouracil alone.

The ability to withstand stress, as measured by the number of cage revolutions necessary to exhaust the birds, was reduced by feeding thiouracil and was increased by adding thyroprotein to diets containing thiouracil. Protamone, at the level of 44 ppm., increased stress resistance markedly. It was also noted that, at all treatment levels, the male birds showed a generally greater resistance to exhaustion than did the females.

The results of the tests involving sex hormones and stress are shown in Table 3. Stress reduced body weight in all groups. This reduction was especially marked in the presence of androgen and estrogen. The effects of the various hormones on body weight were unchanged by stress. Both androgen and estrogen caused an increase in weight above that of the uninjected groups.

A reduction in thyroid size was noted in the presence of androgen injection and stress. Without stress, the hormonal injections had no effect on thyroid size.

In the presence of androgen, adrenal size was sharply increased by stress. This was especially true at the 1.4 mg. level of androgen. An increased adrenal size was also noted with stilbesterol treatment, whether the birds were stressed or not.

The gonads of both sexes were decreased in size by androgen and estrogen. The combs of both sexes responded to androgen with a marked increase in size. Neither of these effects was altered by stress.

Resistance to exhaustion was markedly greater in the groups receiving androgen than in the untreated controls. However, the greatest resistance to stress was shown by the gonadotropin-treated birds. This is in contrast to the lack of effect of gonadotropin on the other factors measured.

The effect of stress on the histology of the adrenal is illustrated in Figure 1. A complete disruption of the normal tissue structure of this gland was seen after stress, with the medullary islets being invaded and disrupted by rapidly proliferating cortical tissue. These changes occurred regardless of whether a high degree of resistance to fatigue was recorded or not. In all cases, stress caused an increase in adrenal gland size. This increase was apparently due to hypertrophy and hyperplasia of both cortical and medullary tissues.

TABLE 3. EFFECT OF SEX HORMONES, GONADOTROPIN, AND STESS ON THE BODY AND ORGAN WEIGHTS OF CHICKS

	0.7 mg. androgen	1.4 mg. androgen	0.7 mg. estrogen	70 units gonadotropin	Control (no injection)
Body, gm.					
Stress	252 ± 11	266 ± 11	272 ± 7	235 ± 18	249 ± 19
No stress	292 ± 10	301 ± 14	303 ± 13	264 ± 8	273 ± 10
d[1]	40	35	31	29	24
Thyroid, mg.					
Stress	11 ± 2	13 ± 1	16 ± 1	13 ± 2	16 ± 2
No stress	17 ± 2	17 ± 1	18 ± 3	15 ± 2	16 ± 2
d	6	4	2	2	0
Adrenal, mg.					
Stress	41 ± 3	47 ± 2	46 ± 3	39 ± 2	44 ± 4
No stress	34 ± 1	37 ± 2	45 ± 2	38 ± 2	37 ± 2
d	7**	10**	1	1	7
Testes, mg.					
Stress	47 ± 40	48 ± 5	38 ± 3	80 ± 12	68 ± 9
No stress	46 ± 0	47 ± 6	49 ± 10	74 ± 12	115 ± 24
d	1	1	11	6	47
Ovary, mg.					
Stress	39 ± 3	42 ± 6	50 ± 3	50 ± 3	53 ± 6
No stress	49 ± 3	42 ± 6	47 ± 6	54 ± 5	60 ± 4
d	10	0	3	4	7
Male comb, mg.					
Stress	716 ± 127	820 ± 52	97 ± 29	209 ± 26	166 ± 9
No stress	963 ± 516	918 ± 132	128 ± 12	152 ± 39	488 ± 60
d	247	98	31	57	282**
Female comb, mg.					
Stress	315 ± 17	576 ± 109	63 ± 8	62 ± 10	—
No stress	608 ± 69	551 ± 181	77 ± 7	77 ± 12	71 ± 5
d	293**	25	14	15	—
Heart, gm.					
Stress	1.9 ± .1	1.8 ± .1	1.8 ± .1	1.5 ± .1	1.8 ± .1
No stress	1.7 ± .1	1.8 ± .1	1.9 ± .1	1.6 ± .1	1.8 ± .1
d	0.2	0	0.1	0.1	0
Revs. to exhaust	1168	1308	1034	1403	900
Range	520–2190	345–2370	255–2085	210–2640	195–2080

[1] "d" indicates difference between means for stressed and nonstressed birds within treatments.

** Indicates statistical significance at the 1.0% level.

DISCUSSION

Altered thyroid activity. The body weight of birds treated with thiouracil plus thyroprotein did not equal that of untreated birds regardless of the fact that thyroprotein had returned their thyroxine level to normal or above normal. This may be due to the toxicity of thiouracil.

It is apparent from the results obtained that the two general physiological functions of thyroprotein—TSH opposition and metabolic stimulation—are unequal in degree. This explains the observation that birds fed thiouracil plus small amounts of thyroprotein had larger thyroids than birds fed thiouracil with no thyroprotein. The body weight of birds fed thiouracil was greatly increased when as little as 11 ppm. of thyroprotein was added to the diet. At low levels, the metabolic-stimulating activity

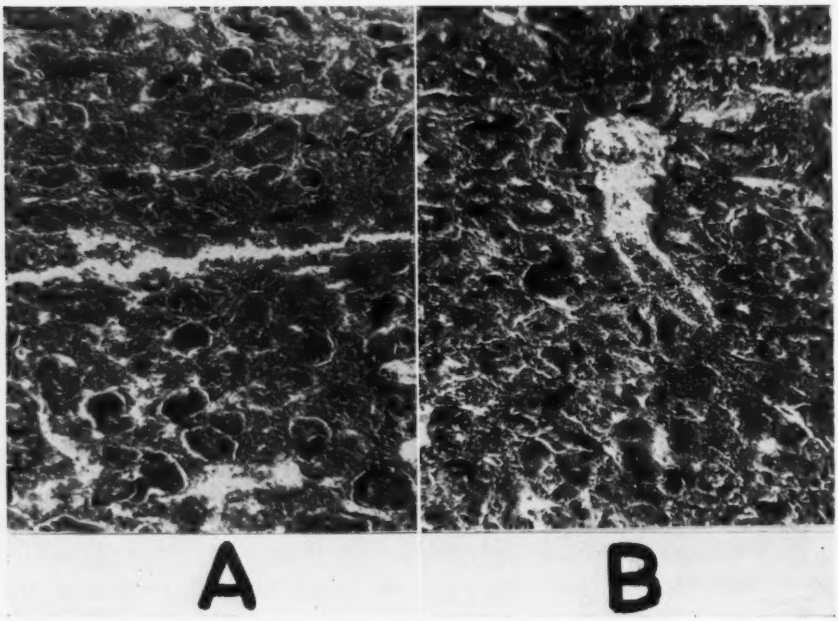

FIG. 1. Representative low power (100X) photomicrographs of adrenals of chicks in the tests. A. Untreated control. Note medullary islets. B. Adrenal from chick which was stressed while on unsupplemented diet.

of thyroprotein is proportionally greater than its TSH-opposing activity. At higher thyroprotein levels, the condition is apparently reversed. The metabolic activity of thyroprotein appears to "plateau," while the TSH-opposing activity continues to increase.

This effect has been noted before. Moreng and Shaffner (1949), using 12-week-old New Hampshire chicks, found that adding 22 ppm. Protamone and 0.2% thiouracil to the ration resulted in a median thyroid weight that was almost twice that of the group fed 0.2% thiouracil alone. More recently, Sellers *et al.* (1953) have reported that 0.02% propylthiouracil plus 0.02% thyroid extract fed to rats over long periods caused a great increase in thyroid size over that seen in animals on the goitrogen alone. These authors believe that the thyroid hormone supplied as dried thyroid in the diet was less than necessary to inhibit the pituitary, and a "hormonal imbalance" resulted. In view of the results reported in the present paper, it is likely that this condition exists only at low thyroid hormone levels.

Extreme hypothyroidism inhibited comb growth even more than would be expected from the concurrent decrease in body size. Testis size was not

markedly reduced. This indicates that the development of the comb depends upon normal thyroid activity as well as upon androgen activity.

Neither the adrenal glands nor the gonads were markedly changed in size by variations in thyroid activity, except in the presence of severe hypothyroidism. The activity of these organs, as measured by their weight, is not affected by fairly great fluctuations in thyroid activity.

Thyroid activity and stress. Garren and Shaffner (1953) have shown that the adrenal gland of the normal chick enlarges in response to exercise stress. It is apparent from the results listed in Table 2 of the present paper that the adrenal of the chick does enlarge markedly in response to stress, and that this enlargement is not greatly affected by variations in thyroid activity. Normal thyroid secretion is not essential for greatly increased adrenal size and activity, although severe hypothyroidism reduces somewhat the ability of the adrenal to enlarge after stress (Table 2, group 2; thiouracil only).

It was noted that all birds which resisted stress well had greatly enlarged adrenals, but of the birds which failed to resist stress not all lacked adrenal enlargement. Thus adrenal enlargement *per se* was not a guarantee of a successful response to stress.

It is to be emphasized that marked adrenal enlargement occurred even in those groups receiving little or no thyroprotein, i.e., those birds with greatly enlarged thyroids. The pituitary glands of these chicks were, therefore, able to produce large quantities of TSH and ACTH simultaneously. In addition, gonad weights were not decreased in those groups which received thiouracil plus small amounts of thyroprotein and were stressed. In those groups receiving thiouracil and stress, the reduction in gonad size was closely correlated to reduced body size. In view of these results, as well as those obtained without stress, it appears that in the chick greatly increased secretion of one anterior pituitary hormone does not constitute a shift in hormonal output at the expense of the other anterior pituitary hormones, but that the secretion levels of these other hormones may concurrently remain normal or even increase in level. Since this shift does not occur, it seems possible that the control of pituitary activity by the hormones of the target glands may occur outside of the pituitary itself. If the control were exerted directly upon the cells of the pituitary, it would be reasonable to expect that variations in the output of any one hormone might affect that of the others.

The results also show that, at some levels, thyroxine and adrenal hormones may act together to cause gonadal enlargement. A statistically significant increase in ovarian weight was observed at the 22 ppm. level of thyroprotein when stress was induced. This may be due to a gonadotropic action of adrenal secretions at a favorable thyroxine level.

Although the stress was severe enough to induce considerable adrenal enlargement, significant cardiac enlargement was noted only in those groups receiving thiouracil and no thyroprotein. Thus the thyroid deficiency of young chicks must be severe before a concurrent general stress may induce cardiac damage.

Sex hormones and stress. In general, the chicks responded to androgen and estrogen in the expected manner. Often this response was not altered by stress. Although the average body weights of all animals in these tests were depressed by exercise, the differences in weight between the stressed and non-stressed groups were generally not statistically significant. It is noted, however, that the depression is greater than that which was observed with variations in thyroxine level and stress.

Within the groups which were treated with androgen, stress reduced thyroid size. This reduction approached statistical significance indicating that there may be an action by androgen and the adrenal hormones on the production of TSH.

It is also noteworthy that the expected adrenal enlargement after stress occurred in the presence of androgen, but not in the presence of estrogen or gonadotropin. It appears, however, that stilbestrol itself may have elicited some adrenal enlargement.

The results show that increased androgen activity may aid in attaining a successful resistance to stress. This is especially true in the female. The mode of action of this gonadotropin sample whereby it increased resistance to stress, while inhibiting adrenal enlargement, is unknown.

SUMMARY

Studies were made on the effect of variations in thyroxine and sex hormone levels on normal chicks and on chicks subjected to the stress of muscular fatigue. Small amounts of thyroprotein were found to have a relatively greater metabolic-stimulating effect than the TSH-opposing effect. At higher levels this condition was reversed.

Stress caused a marked adrenal enlargement, due to increase of both medullary and cortical tissue, which was generally unaffected by variations in thyroxine or gonadal hormone levels. This enlargement was indicative of but not necessarily allied with a successful resistance to stress. Since adrenal enlargement occurred in goitrous chicks exposed to stress, without concurrent gonadal atrophy, and in androgen-treated birds which were stressed, the pituitary does not necessarily shift its activity in response to increased demand for one of its hormones.

Androgen increased the ability to resist stress somewhat more in females than in males. Untreated males resisted stress better than comparable females, and thyroprotein increased the resistance in both sexes.

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THE GROWTH OF THYROIDS AND ADRENALS IN THE CHICK

W. R. BRENEMAN[1]

Department of Zoology and Waterman Institute, Indiana University[2]

THERE is no detailed account of normal thyroid or adrenal growth over any considerable period of the life span of any vertebrate. The basic physiologic interactions involved in the thyroid-pituitary and the adrenal-pituitary axes have been determined by experimental studies and although these investigations have established clearly the primary role of the pituitary tropic hormones in thyroid and adrenal function, there are certain problems of glandular inter-relationships which can be analyzed very profitably by a study of the growth of normal glands. The data on normal animals provide an important complement to experimental observations because it can be ascertained:

1. Whether sexual differences exist in gland sizes or growth rates.
2. If sexual differences are observed, these can be compared with the physiological changes which occur during the development of the reproductive system.
3. Whether the rate of growth of one gland is comparable with that of another.
4. The approximate time at which the secretion of the various pituitary tropic hormones is initiated.

Furthermore, the comparisons which are possible in an investigation of normal animals are especially valuable because they permit an evaluation of a time factor, that is, the age at which various specific endocrine events occur. Finally, the possibility that abnormal physiological conditions have been produced by experimental treatment does not exist.

It is the primary intent of this paper to record the weight changes in thyroids and adrenals of White Leghorn chicks from hatching until 140 days of age, when the birds have reached sexual maturity and have obtained most of their body growth. The four points as outlined above will be considered at some length.

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MATERIALS AND METHODS

Single comb White Leghorn chicks were used exclusively in this study. The birds were approximately 24 hours old when received in the laboratory and were placed in battery brooders at 48 hours of age. Care was taken to avoid crowding and the number of chicks per cage was reduced as the animals became older. Initially each cage contained 100 chicks, but the number was reduced to at least 12 at 90 days. Larger finishing batteries which provided more head room also were used after about 50 days of age. All birds were wing-banded and individual records were kept.

Size increases in adrenals and thyroids also were determined for capons. The capons were grown in the same manner as the cockerels and pullets, but were kept in separate cages from the unoperated birds. Caponization was performed on the third or fourth day after-hatching. Ether anesthesia was used and the testes were removed with jeweler's forceps. Since surgery was done with the aid of a binocular microscope, it usually was possible to perform a bloodless operation. Any animal with a trace of testicular tissue at autopsy was considered a "slip" and was excluded even though comb and pituitary size were comparable with those of the capons.

All animals were killed by decapitation, and the glands were quickly removed and weighed on a torsion balance. A total of 1274 cockerels, 568 pullets, and 361 capons was used in the determination of thyroid and adrenal weights. An additional 1458 cockerels and 84 capons, which were part of another study of pituitary and gonad weights also were used here to contribute information for the determination of testicular growth curve and for estradiol and testosterone effects on gland weights. The various curves depicted in the figures were calculated according to statistical methods for curvi-linear regression as outlined by Snedecor (1946).

RESULTS AND DISCUSSION

Growth of thyroids and adrenals. The growth curves for the thyroid glands for different ages are presented in Figure 1. The curves show clearly that there are no significant differences in the average weights of the thyroid gland in capons, males, or females from hatching until 90 days of age. The capon thyroid weights begin to exceed those of the cockerels and pullets at 90 days after hatching and at 110 days, the thyroids of males also average appreciably more than those of females. The curves continue to diverge and at the termination of observations, the weight differences are substantial. The most noteworthy fact, however, is the remarkable uniformity of growth previous to 90 days in these three physiologically diverse groups of chicks.

Two other observations relative to the curves of thyroid growth should also be noted. First, the weight increases begin immediately after hatching and continue at a rather constant rate until the 50th or 60th day. At this time, the rate of growth is accelerated especially in capons and cockerels. This period would appear to mark the time at which the pituitary tropic hormone is most effective. Second, in the interval between 100 and 120 days of age there is a slowing of the growth rate and this is more pronounced in cockerels and pullets than it is in capons.

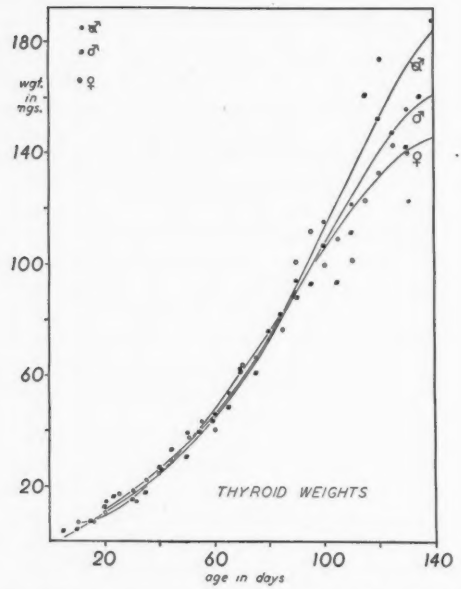

FIG. 1. *Growth of chick thyroid glands.* The mean weights of the glands are plotted on a uniform scale for the various ages of White Leghorns up to 140 days of age. All seasons of the year are represented in the samples.

Although the growth curves for the adrenal glands which are presented in Figure 2 are in marked contrast in several respects from the growth curves for the thyroid glands, there are similar weight increases for the two glands immediately following hatching. There is, however, an obvious difference between the average weights of the adrenals of the capons and cockerels and those of the pullets as early as the 30th day of age and these weight differences become more pronounced as the animals increase in age. The adrenal weight averages of capons and males, on the other hand, do not differ significantly until after 100 days of age.

There are additional differences in the adrenal growth curves which are significant. First, the growth rates decrease during the period between 100 and 120 days of age. Second, this decline in the rate of growth is obvious in capons and pullets, but is less evident in males. Third, it should be observed that, in contrast to the thyroid weight, the male adrenal weight averages exceed those of the capons in the older birds.

Limited data for the growth of certain endocrine glands and viscera in the chick for the first 30 days after hatching were presented earlier. Breneman (1941), and in this study the data were recorded as percentages of body weight. This relationship had considerable merit in the study

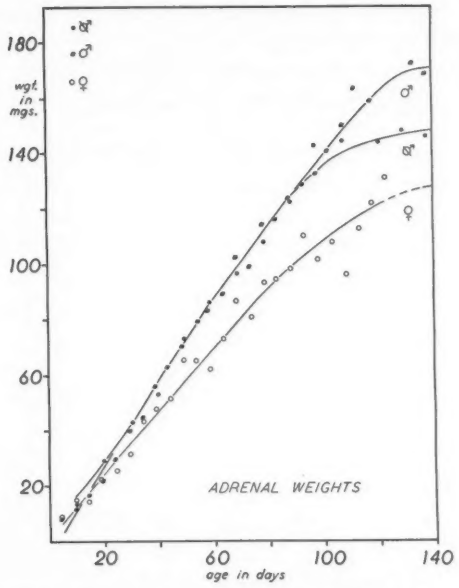

FIG. 2. *Growth of chick adrenal glands.* The mean weights of the glands are plotted on a uniform scale for the various ages of White Leghorns up to 140 days of age.

because it was possible to compare gland weights of White Leghorn and Rhode Island Red chicks. The expression of gland weights as percentages of body weight also has been advantageous when experimental animals of the same ages are compared. The range of individual variations is usually decreased and slight discrepancies due to the differences in the body size of birds in different series are adjusted to a uniform standard. It did not seem to be justified in the present investigation, however, to express thyroid and adrenal weights as percentages of body weight and the reasons may be summarized briefly. First, the ages of the birds extended over a period of more than $4\frac{1}{2}$ months; the physiological changes during this period are very extensive and are as yet imperfectly understood; third, there is little knowledge of the interactions between the various glands of the chick during this period of rapid growth and development.

It does appear important, however, to compare the relative growth rates of the thyroid and adrenal glands in somewhat more detail than was done in Figures 1 and 2. Furthermore, since sex differences were observed in the growth rates of both glands, comparisons with the growth of the gonads was suggested. For this reason, the curves for thyroid and adrenal growth of the cockerels are presented together with the changes in testis weights in Figure 3. The comparison is limited here to cockerels although

similar data are available for analysis of pullets. It is possible in Figure 3, therefore, to visualize the weight changes which occur in thyroids and adrenals of males at all time periods, and in addition, to compare the growth of these two glands with the size increases of the testes.

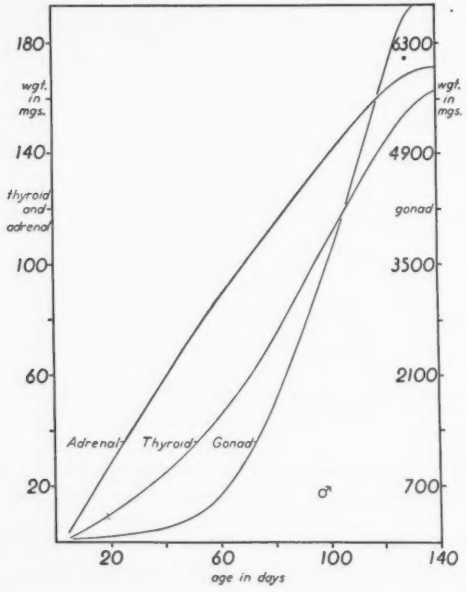

FIG. 3. *Comparative growth rates of thyroids, adrenals, and gonads in cockerels.* The mean weights of thyroids and adrenals are plotted on the same scale, gonad weights are plotted on a different scale but with the same number of subdivisions of scale as used for the other glands.

The growth rates of the three glands are very different. Adrenal weight increases begin at hatching and continue at a remarkably constant rate until near the termination of observations. Gonad weight increments, in contrast, are appreciable but gradual from hatching until 60 days of age at which time the growth rate increases tremendously. It is not until about 120 days that a slower rate of testis growth begins. Thyroid weights, on the other hand, tend to be intermediate between those of adrenals and gonads; the most notable features of the thyroid growth curve being the marked increase which was previously noted at 50 days of age and the somewhat slower rate of increase after about 100 days of age.

It may be questioned if the weight increases of the chick thyroids, adrenals, and testes, are a reflection of the secretion of pituitary tropic hormones. The data presented here do not provide any estimate of the

amount of pituitary hormones which may be released, but experimental data for the presence of tropic hormones in chicks is rather extensive. The evidence for the presence of thyrotropin (TSH) is excellent and is both direct and indirect. Assays of chick pituitaries for TSH have been made by Morris (1952). Large goiters will develop in chicks which are fed thiouracil and Odell (1952 a and b) has reported that the goiterogenic action of thiouracil is counteracted by the administration of thyroxin. Thyroxin also will inhibit thyroid growth in chicks, presumably by inhibiting pituitary secretion of TSH. The pattern of responses, therefore, is the same as that in mammals. Finally, Smelser (1937, 1938) and Albert *et al.* (1946) and Albert (1949) have shown that the chick thyroid is very sensitive to small amounts of thyrotropic hormone and work in this laboratory by Tolman (unpublished data) has demonstrated that as little as 0.05 International Units of TSH produces significant increases in weight and cell height of chick thyroids.

Although relatively little is known about the secretion of adrenocorticotropin (ACTH) in chicks, Jailer and Boas (1950) have reported that the administration of epinephrine is accompanied by increased adrenal weights in chicks probably as a result of the release of ACTH from the pituitary. Dulin (1952) verified this and also demonstrated that this weight increase is prevented if cortisone is injected simultaneously with epinephrine. As little as 5.0 μg. of an ACTH preparation (Armour—3.65 I.U. mg.) produced significant adrenal weight increases in the 3-day old chick test. Dulin also was able to stimulate adrenal growth by the administration of 4 μg of 40-day old chick pituitary into 3-day old test animals. The increase was slightly greater than that produced by 50 μg. of the ACTH preparation. Except for the report of Jailer and Baos that the ascorbic acid content of the adrenal cortex was not depleted by ACTH injection, the relationship between the anterior pituitary and the adrenal cortex of chicks appears to be comparable with that of the mammals.

It has been possible in previous studies, Breneman (1944, 1945), to establish that there is a considerable release of gonadotropic hormone from the chick pituitary at an early age after hatching. This secretion has been quantitatively determined and is found to vary quite considerably with the administration of sex hormones. The data reported by Breneman and Mason (1951) has also demonstrated that considerable difference exists between the pituitary gonadotropins in capons and cockerels. Only total gonadotropin was measured, however, and no information is available at present concerning the relative amounts of follicle stimulating or interstitial cell stimulating hormones which may be present.

It seems logical, therefore, to assume that much of the variation in weight increments which are observed for the three glands shown in

Figure 3 must indicate the effects of the pituitary tropic hormones on the adrenals, thyroids, and testes. It is apparent, however, that differences also must exist either: in the rate of tropic hormone secretion; in the ability of glands to respond; or to a combination of both factors. At present there is only limited information relative to these three variables, but it seems probable that the thyroids and possibly also the adrenals are more sensitive to their respective tropic hormones than are the testes. The thyroid studies of Smelser, Albert, and Tolman which were previously referred to support this conclusion. It is clear that further analysis of the growth of the thyroid and adrenal glands in the chick is needed to test this hypothesis, and initially a determination of the rate of secretion of the tropic hormones of the pituitary must be made. Assay for the content of TSH and ACTH in the anterior pituitary glands of chicks of various ages is now in progress. This should afford an opportunity to ascertain the relative importance of the amount of the tropic hormone secretion and the ability of the glands to respond.

Effect of gonadal hormones. Since sex differences were observed in the growth of the adrenals and thyroids and because the increments of the capon glands differed from those of pullets and cockerels, it was essential to determine what effect male and female sex hormones would have on the weights of the glands. For this reason, doses of estradiol-dipropionate were administered from the 20th to the 39th day of age to two groups of animals and autopsies were performed at the 40th day. Estradiol-dipropionate only was given to capons and to one series of males, and in addition, a second group of males received 20 μg. daily of testosterone-propionate with each dose of estradiol-dipropionate. These data were part of a more extensive analysis of estrogen and androgen effects on the pituitary-gonad axis of the chick (Breneman and Mason, unpublished data). The thyroid and adrenal weights are presented in Tables 1 and 2 and the data are treated statistically by an analysis of variance according to the method outlined by Snedecor (1946). This makes it possible to analyze the significance of differences between the means of each group of animals and of the differences of means within each group.

The data presented in Tables 1 and 2 as "source of variation—capons and males" demonstrates that the thyroid and adrenal weights of treated capons and males do not differ significantly. These results could be anticipated from the data for 40-day old animals presented in Figures 1 and 2 and it was gratifying to have this additional confirmation in experimental animals. The thyroid weights for the seven levels of estradiol-dipropionate treatment have six degrees of freedom alloted to them and the differences as measured by the F-test slightly exceed the 5% level of significance. An examination of the means for the different treatments, however, shows

TABLE 1. ANALYSIS OF THYROID WEIGHTS OF CAPONS AND MALES WHICH RECEIVED GONADAL HORMONES[a]

Experimental groups	Daily dosage of estradiol dipropionate[b]							Experimental groups Sums and means		
	0	0.2γ	1.0γ	2.0γ	10.0γ	20.0γ	40.0γ	No.	Sum	Mean
Capons No.	13	10	12	12	11	12	12	82		
Sum of thyroid weights at each dosage level	262	243	285	303	230	256	239		1818	
Mean thyroid weights	20.15	24.30	23.67	25.25	20.91	21.33	19.92			22.17
Males No.	14	14	14	13	14	14	14	97		
Sum of thyroid weights at each dosage level	345	352	304	285	296	339	300		2221	
Mean thyroid weights	23.64	25.14	21.71	21.92	21.14	24.21	21.43			22.90
Males given 20 μg. of testosterone propionate[c] daily	14	14	14	14	14	14	14	98		
Sum of thyroid weights at each dosage level	262	312	269	322	288	309	340		2102	
Mean thyroid weights	18.71	22.28	19.21	23.00	20.57	22.07	24.28			21.45
No.	41	38	40	39	39	40	40	277		
Sums for three groups at each dosage level	869	907	858	910	814	904	879		6141	
Means for three groups at each dosage level	21.20	23.86	21.45	23.33	20.87	22.60	21.98			22.17

Analysis of variance

Source of variation	Degrees of freedom	Variance	Mean squares	F value
Capons and Males	2	102	51.00	2.50
Estradiol	6	296	49.33	2.42*
Error	268	5461	20.38	

[a] All weights are in milligrams.
[b] Courtesy of Dr. Robert Gaunt, Ciba Pharmaceutical Products, Summit, New Jersey.
[c] Courtesy of Dr. Edward Henderson, Schering Corporation, Bloomfield, New Jersey.
* Indicates a 5% level of significance.

that there is no pattern of response which can be correlated with an increasing or decreasing amount of hormone. Reference to either Figures 1 or 3 will demonstrate that the weights of control thyroids (0 estradiol-dipropionate) are somewhat lower than the 40-day control weight average. This slightly atypical control series, therefore, probably is primarily responsible for the differences as measured by the F-test.

The data for adrenal weights follow the same pattern as do those for the thyroids and again there is no significant difference between the adrenal weights of capons and males. The F-test of the adrenal differences for the estradiol-dipropionate treatments barely has a 5% level of significance as was the case with the thyroids. Reference to either Figure 2 or 3 shows that the control adrenal weights in this series are also slightly below the average for the 40-day birds. Since the F value only exceeds slightly the 5% level and since there are no consistent differences in the weights of the adrenals which can be correlated with different hormone levels, it is felt that, as in the case of the thyroids, no significance should be attributed to these differences at present.

It may be questioned whether the dosages of the two steroids administered were adequate to inhibit the production of any of the tropic hormones of the pituitary in this experiment. It was observed (to be reported later) that all gonadotropin secretion is stopped in chicks of this age by dosages

TABLE 2. ANALYSIS OF ADRENAL WEIGHTS OF CAPONS AND MALES WHICH RECEIVED GONADAL HORMONES[a]

Experimental groups	Daily dose of estradiol dipropionate							Experimental groups Sums and means		
	0	0.2	1.0	2.0	10.0	20.0	40.0	No.	Sum	Mean
Capons No.	13	11	13	12	11	12	12	84		
Sum of adrenal weights at each dosage level	626	586	713	618	568	599	589		4299	
Mean adrenal weights	48.15	53.27	54.85	51.50	51.64	49.92	49.08			51.18
Males No.	14	14	14	14	14	14	14	98		
Sum of adrenal weights at each dosage level	759	779	757	702	827	706	832		5362	
Mean adrenal weights	54.21	55.64	54.07	50.14	59.07	50.43	59.43			54.71
Males given 20.0 of testosterone propionate daily no.	14	14	14	14	14	14	14	98		
Sum of adrenal weights at each dosage level	686	763	740	741	748	754	751		5183	
Mean adrenal weights	49.00	54.50	52.86	52.93	53.43	53.86	53.64			52.90
No.	41	39	41	40	39	40	40	280		
Sums for three groups at each dosage level	2071	2128	2210	2061	2143	2059	2172		14,844	
Means for three groups at each dosage level	50.51	54.56	53.90	51.52	54.95	51.43	54.30			53.01

Analysis of variance

Source of variation	Degrees of freedom	Variance	Mean squares	F value
Capons and Males	2	568	284.00	2.27
Estradiol	6	1,790	298.33	2.39*
Error	271	33,892	125.06	

[a] All weights are in milligrams.
* Indicates a 5% level of significance.

of 6 μg. or more daily of estradiol-dipropionate. It must be concluded, therefore, that at these dosage levels the effect of estradiol on TSH and ACTH secretion is very different from its effect upon the release of gonadotropin.

Seasonal variation of thyroid weights. Albert *et al.* (1946), have pointed out that one of the difficulties in the assay of TSH may be a seasonal variation in the thyroid size of assay animals. Since it is known that extremes of temperature influence the production of thyroid hormone. There is an opportunity in this study to check this source of variation in young normal animals. The temperatures in the laboratory do not vary as much as do those to which chicks would be subjected if they were allowed to live on open range. There is occasional seasonal variation in temperatures, however, which cannot be controlled adequately. Temperatures on a few occasions have dropped to the low sixties during the winter for a few hours and likewise have reached the high eighties in the summer. The analysis of this problem was somewhat complicated by the fact that chicks are not available in adequate numbers at all seasons of the year. Accordingly, the number of birds in the various age groups were not equally represented nor were all age groups studied. The data in Table 3, however, present a fairly representative sample. The winter (cold) season includes only the months of November through February, the summer (warm) season only the

months of May through August. An analysis of variance was again used to test the significance of the differences between means.

The analysis of variance for the samples selected for Table 3 shows clearly that no significant seasonal variations existed in thyroid weights in this study. The F value of 282 is far short of even the 5% level of significance. The 105-day old summer birds had thyroids which were heavier than those of the winter birds and some additional limited data indicate that there may be a real seasonal difference in these older animals. This conclusion must be tentative, however, until additional series are studied.

TABLE 3. ANALYSIS OF SEASONAL VARIATION IN THYROID WEIGHTS[a]

	Age						Seasonal sums and means		
	25	30	35	40	50	105	No.	Sum	Mean
May to Aug.									
No.	27	36	39	12	12	31	157		
Sums of thyroid weights at each age	335.07	642.18	1038.18	409.00	587.00	3016.00		6028.04	
Mean thyroid weights	12.41	17.86	26.62	34.08	48.92	97.23			38.40
Nov. to Feb.									
No.	11	11	19	19	14	9	83		
Sums of thyroid weights at each age	103.18	162.50	533.00	632.00	690.00	826.00		2946.68	
Mean thyroid weights	9.38	14.77	28.05	33.26	49.28	91.78			35.50
No.	38	47	58	31	26	40	240		
Sums for age groups	438.25	805.29	1571.18	1041.00	1277.00	3842.00		7974.72	
Means for age groups	11.53	17.13	27.09	33.58	49.12	96.05			37.39

Analysis of variance

Source of variation	Degrees of freedom	Variance	Mean squares	F value
Season	1	542	542	2.82
Age	5	192,472	38,494	199.99**
Error	233	44,850	192.48	

[a] All weights are in milligrams.
** Indicates 1% level of significance.

SUMMARY AND CONCLUSIONS

Size increases in chick thyroid glands are apparent immediately after hatching and the rate of growth increases notably at about 50 days of age. There are, however, no significant differences in the weights of thyroids of males, pullets, or capons until about 90 days of age, after which time the capon glands exceed in size those of the males which in turn are larger than those of the females. The rate of growth of the thyroids declines in all three groups of animals after about 100 days after hatching. Adrenal increments are remarkably uniform and the adrenal weights of capons and males exceed those of females as early as 30 days after hatching. After 100 days of age, male adrenal weights are heavier than those of capons.

A comparison of the growth curves for thyroids, adrenals, and gonads in cockerels shows that the growth rates for the three glands are very different. The relative growth of the testes is slowest previous to 60 days

after which time the growth is greatly in excess of that of either the thyroids or adrenals.

Administration of estradiol-dipropionate in dosages ranging from 0.2 μg. to 40.0 μg. per day for 20 days either alone or in combination with 20.0 μg. of testosterone-propionate daily had little or no effect on thyroid or adrenal weights in either males or capons. No significant seasonal differences in thyroid weights were noted for cockerels of different ages during the winter and summer months. The data for both the hormone treatments and for the seasonal differences were analyzed according to analysis of variance.

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ESTROGEN ASSAYS USING THE RAT UTERUS[1]

RALPH I. DORFMAN[2] AND ADELINE S. DORFMAN[2]

Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts and the Departments of Biochemistry and Medicine, Western Reserve University School of Medicine and Lakeside Hospital, Cleveland, Ohio

THE use of the spayed rat's uterus as an end-point for estrogen assay was suggested by Bülbring and Burn (1935). Dorfman *et al.* (1936) and Lauson *et al.* (1939) used the uterine response of the intact immature rat for estrogen assay. Neither of these studies nor the studies of Astwood (1938) who described a 6-hour test and also used the intact immature rat uterus contained statistical analysis of the data. Emmens (1950) calculated the precision of the data of Lauson *et al.* (1939) and found that if a total of 40 rats are used in a 4-point assay, the expected minimal limits of error are approximately ($P = 0.95$) 81 to 124%.

This report was designed to study the utility of immature rat uteri as an end-point for the quantitative bioassay of estrogens using both the subcutaneous and oral routes of administration and to study the relative oral activity of various estrogen sulfates.

ANIMALS AND MATERIALS

The albino rats employed in this study were obtained from Carworth Farms. They were 22 to 23 days of age at the start of the experiments.

Two samples of sodium estrone sulfate were used, one was a synthetic sample, m.p. 229–232° C. (sample A) and the second was a sample prepared from impure estrone isolated from pregnant mare's urine (sample B). Other estrogen conjugates included sodium equilin sulfate, a reduced estrone sulfate preparation (estradiol-17β sulfate), and a reduced equilin sulfate preparation (dihydro-equilin sulfate).[1]

EXPERIMENTAL DETAILS

For studies involving the subcutaneous injection of estrogens the 22 to 23-day-old rats were injected once daily for three days with 0.1 ml. of corn oil solutions of the hormone. The uteri were removed 24 hours after the last injection, the fluid removed by blotting on filter paper, and weighed to 0.5 mg. Body weights were determined and the results expressed as uterine ratios which are defined as 100 times the weight of uterus in mg. per gram of body weight.

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[2] Present address: Worcester Foundation for Experimental Biology.

For studies involving the oral administration of estrogens the 22 to 23-day-old rats received the standard and unknown by stomach tube once daily for 4 days. The daily dose of steroid was contained in 1 ml. of water. The estrogens were stored in 95% ethanol solutions and kept in the refrigerator at 4° C. for periods not longer than one week. The aqueous solutions were made fresh each day within one-half hour of use. Twenty-four hours after the last oral administration, the rats were autopsied, at which time body and uterine weights were determined. The results were expressed in terms of uterine ratio.

The Bliss (1944) method was used for determining the potency ratio, the standard error of the potency ratio, and the significancy of difference of the slopes of the regression lines of the standard and unknown. The statistical methods of Fischer (1934) and Bülbring (1937) were also employed.

RESULTS AND CONCLUSIONS

Oral Administration

The results of uterine stimulation by increasing doses of sodium estrone sulfate in the rat are presented in Table 1. As little as 2.5 μg. of sodium

TABLE 1. RAT UTERINE RESPONSE TO ORALLY ADMINISTERED SODIUM ESTROGEN SULFATES

Preparation	Total dose, μg.	Number of rats	Mean uterine ratio ±S.E.
0	0	11	54 ± 3.7
0	0	13	56 ± 1.6
Sodium Estrone Sulfate (Sample A)	2.5	17	77 ± 5.7
	5.0	22	108 ± 9.4
	10.0	32	141 ± 7.8
	20.0	11	184 ± 12.3
Sodium Estrone Sulfate (Sample B)	4.0	13	67 ± 2.6
	8.0	12	104 ± 8.9
Reduced Sodium Estrone Sulfate	2.0	12	86 ± 8.0
	4.0	14	103 ± 5.4
Sodium Equilin Sulfate	4.0	10	75 ± 2.6
	8.0	17	108 ± 5.2
	16.0	10	144 ± 3.8
	32.0	9	188 ± 9.3
Reduced Sodium Equilin Sulfate	2.0	9	151 ± 6.0
	4.0	17	191 ± 4.6
	8.0	9	196 ± 5.8
	16.0	7	216 ± 10.2

estrone sulfate (equivalent to 2.0 μg. of estrone) produced a significant increment in uterine weight. Between 2.5 and 20 μg. of estrone, four logarithmic intervals, a straight line function was established on the basis of the log dose-response relationship.

Using the Bliss design, a series of test assays were performed where sodium estrone sulfate (sample A) was compared to itself (Table 2). Here

TABLE 2. THE ORAL ASSAY OF SODIUM ESTRONE SULFATE USING THE RAT UTERUS

$$\frac{\text{High Dose Standard}}{\text{Low Dose Standard}} = \frac{\text{High Dose Unknown}}{\text{Low Dose Unknown}}$$

Standard = Unknown

N	Dosage levels, μg.	b	λ	t	Potency ratio ±S.E.
5	10; 20	106	0.352	2.840	67 ± 28
5	10; 20	179	0.191	0.679	127 ± 26
8	2.5; 5	105	0.117	0.961	104 ± 28
8	2.5; 10	120	0.103	0.332	99 ± 25
10	5; 10	86	0.397	1.142	127 ± 39
10	5; 10	102	0.428	0.246	75 ± 25
20	5; 10	100	0.369	0.539	96 ± 18

N = Number of sets of animals, N × 4 = Total number of animals used for an assay.
b = Combined slopes for standard and unknown.
λ = Index of precision.
t = Fischer's t value for significancy of difference between slopes of unknown and standard.

the theoretical potency ratio should be 100%. When a total of 20 rats was used (N = 5), the comparisons were not satisfactory. In one of the two tests the slopes were significantly different and the potency ratios were 67 ± 28, and 127 ± 26. In the five tests where 32 or more rats were used, the results were considered adequate. Thus, when N was equal to 8 (total animals 32) potency ratios of 104 ± 28 and 99 ± 25 were found and the t values for the slopes were 0.961 and 0.332, respectively. When N was equal to 10, the potency ratios were 127 ± 39, and 75 ± 25 with t values of 1.142 and 0.246, respectively. When a total of 80 rats was used, N equaled 20, and the potency ratio was 96 ± 18 and t for slopes was 0.539.

Table 3 represents the same typical comparisons between pairs of estrogen sulfates. If sodium estrone sulfate is considered to have a potency of 100% then sodium equilin sulfate has a potency of 110%; reduced estrone sulfate, 261%; and reduced equilin sulfate has the highest activity, 972%.

Subcutaneous Injection

The increasing uterine response with increasing dose for subcutaneously administered estrone is represented in Table 4. The log dose-response relationship gave a straight line from 0.6 to 2.4 μg. of estrone. Table 5 indicates a set of trial runs where estrone was assayed against itself. In 10 such trials the maximum deviation (standard error) from the theoretical value of 100% was 22%. In no instance was there a departure from parallelism between the slopes of the standard and unknown.

SUMMARY

Simple methods are described and analyzed statistically for the assay of estrogens by oral and subcutaneous injection using the end-point of

TABLE 3. ASSAY OF ORALLY ADMINISTERED SODIUM ESTROGEN SULFATES

Estrogens assayed		Total dose, μg.	No. of rats	Potency ratio unknown	Error range %
Standard	Unknown			Standard %	P = 0.95
Sodium Estrone Sulfate		4; 8	25	—	—
	Reduced Sodium Estrone Sulfate	2; 4	26	261	−26; +35
Sodium Estrone Sulfate		4; 8	25	—	—
	Sodium Equilin Sulfate	4; 8; 16; 32	46	110	−15; +18
Sodium Equilin Sulfate		4; 8; 16; 32	46	—	—
	Reduced Sodium Equilin Sulfate	2; 4	26	882	−15; +18

TABLE 4. RESPONSE OF THE IMMATURE RAT UTERUS ESTRONE

(Rats, 22–23 days of age, were injected subcutaneously once daily with solvent or estrone in 0.1 ml. of solvent. Autopsy was performed 24 hours after last injection)

Total dose estrone, μg.	No. of rats	Mean uterine ratio ±S.E.
0	20	56 ± 1.1
0.6	16	74 ± 2.7
1.2	16	92 ± 3.1
2.4	16	120 ± 4.5

TABLE 5. ASSAY OF ESTRONE (SUBCUTANEOUS INJECTION)

$$\frac{\text{High Dose Standard}}{\text{Low Dose Standard}} = \frac{\text{High Dose Unknown}}{\text{Low Dose Unknown}}$$

Standard = Unknown

Dose levels, μg.	N	b	λ	t	Potency ratio ±S.E.
2.4 :4.8	8	91.3	0.126	1.232	111 ± 11
1.2 :2.4	8	54.3	0.168	0.434	107 ± 15
0.6 :2.4	8	45.7	0.241	0.176	100 ± 20
0.6 :2.4	8	37.3	0.204	0.650	108 ± 18
0.6 :1.2	8	60.4	0.216	0.432	122 ± 22
0.6 :2.4	8	76.2	0.195	1.172	103 ± 16
1.2 :4.8	8	98.1	0.118	0.487	93 ± 9.0
1.2 :2.4	8	105.0	0.093	1.975	105 ± 8.0
1.2 :2.4	8	92.0	0.166	0.814	97 ± 13
0.35:0.70*	9	92.1	0.122	0.591	96 ± 8.4

* These data were collected from a different strain of albino rats in 1936.

uterine weight of the intact immature rat. Sodium estrone sulfate and sodium equilin sulfate have essentially the same activity when orally administered. Reduced sodium estrone sulfate has 261% the activity of

the sodium estrone sulfate while reduction of sodium equilin sulfate resulted in an increased activity of 882%.

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THE INFLUENCE OF CORTISONE AND INSULIN ON THE RESPIRATORY ACTIVITY OF RAT ADIPOSE TISSUE *IN VITRO*[1]

SIDNEY BREIBART[2] AND FRANK L. ENGEL

Departments of Physiology and Medicine, Duke University, Durham, North Carolina

IN EARLIER reports (Engel and Scott, 1950, 1951, Engel, 1951) the effects of insulin and the adrenal cortex on the concentration of glycogen in adipose tissue in the rat were described. Insulin was found to increase the glycogen content of the interscapular adipose tissue of the 48-hour fasted rat both in the fasting and fed states, confirming the previous findings of Wertheimer and his colleagues (Wertheimer and Shapiro, 1948), Fawcett (1948) and Renold *et al.* (1950). Under comparable circumstances, corticotropin, cortisone and various adrenal extracts had no detectable effect on adipose tissue glycogen. However, if insulin were administered to rats pretreated with ACTH or cortisone for 24–48 hours an increase in adipose tissue glycogen occurred which significantly exceeded that from insulin alone. The same exaggeration of the glycogen response to insulin occurred in rats stressed by cold exposure and in adrenalectomized rats receiving a large but constant dose of cortisone. The mechanism by which hyperadrenalcorticism and cold exposure seemingly potentiated this response to insulin in adipose tissue was not elucidated. However, it was speculated in keeping with the earlier suggestion of Wertheimer (Wertheimer and Shapiro, 1948) that the accumulation of glycogen in adipose tissue under most circumstances might reflect the conversion of carbohydrate to fat in this tissue, with the possibility that glycogen accumulation occurred whenever the rate of entrance of glucose into the cell exceeded its rate of conversion to fat. It is well known that insulin promotes both the passage of glucose into the cell (Levine *et al.*, 1950) and lipogenesis from carbohydrate (Stetten and Klein, 1946) while it has been shown more recently that adrenal hormone inhibits lipogenesis (Welt and Wilhelmi, 1950, Brady *et al.*, 1951). The possibility was suggested (Engel, 1951) that adrenal hormone might inhibit lipogenesis from carbohydrate more than insulin

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stimulates the passage of glucose into cell, or its initial phosphorylation, and hence in the presence of insulin glucose might accumulate to a greater degree as glycogen in adipose tissue. This suggestion seems reasonable as long as there is evidence that the stimulation of lipogenesis by insulin cannot be explained solely by the increased uptake of glucose by the cell (Wilhelmi, 1953). Although admittedly highly speculative, it was thought that information concerning this possibility might be derived from the measurement of the R.Q. of adipose tissue of normal and cortisone-treated rats *in vitro* in the presence of glucose and insulin, separately and in combination. A high rate of lipogenesis from carbohydrate would be reflected in an R.Q. exceeding 1.00, while a depression of lipogenesis would result in a lowering of the R.Q. While this work was in progress, Haugaard and Marsh (1952) reported that insulin increased the oxygen consumption of adipose tissue *in vitro* in the presence of glucose and various carbohydrate intermediaries but did not effect the R.Q., and Hills and Stadie (1952) reported that insulin increased the R.Q. of mammary gland slices of lactating rats when the medium contained glucose and acetate. Balmain, French and Folley (1950) had previously reported a similar increase in the R.Q. of this tissue *in vitro* by insulin. Subsequently, Balmain, Folley and Glasscock (1952) found that cortisone inhibited lipogenesis from glucose and acetate in mammary tissue, an effect which could be overcome by insulin. More recently, the same investigators (Folley, 1953) have found that while large doses of cortisone and corticosterone inhibit lipogenesis, small doses may stimulate it. In the present study it was found that the R.Q. of adipose tissue *in vitro* was increased by the addition of glucose to the medium but that insulin had no effect on the R.Q. in the presence of glucose, confirming Haugaard and Marsh (1952). Cortisone pretreatment, in general, depressed the R.Q. and prevented the increase in R.Q. usually seen when glucose was added to the medium. The increase in oxygen consumption reported by Haugaard and Marsh to occur when insulin was added to the medium could not be confirmed.

METHODS AND MATERIALS

Male albino rats of the Vanderbilt strain weighing between 225–350 gm. and fasted 24 hours were used. Cortisone-treated rats were injected intramuscularly with 5 mg. of a saline suspension of cortisone acetate 48 and 24 hours prior to sacrifice by decapitation.

Following sacrifice the dorsal intrascapular fat pad and the retroperitoneal fat bodies lying in the flanks in both sides were removed and placed in an ice cold medium of composition noted below. The brown adipose tissue was carefully dissected free of white fat, connective tissue and muscle. The adhering peritoneal membrane was stripped from the white fat. Tissues were weighed on a Roller-Smith torsion balance and added to paired vessels which had been kept on ice. Brown fat was generally divided into two pieces

weighing between 50 and 100 mg. White fat was usually taken in its entirety, the fat from one side serving as control and the other added to the experimental vessel. In most cases the amount of tissue from any given animal was not sufficient to allow for duplicate determinations of QO_2 and R.Q., but when possible this was done.

For both cortisone-treated and untreated animals tissue from the same rat were placed in paired vessels, one with and one without glucose. When the effect of insulin was studied, paired vessels containing glucose were used, but insulin was added to only one vessel. Thus, each animal served as its own control. The medium used had the following composition: 0.040 M sodium phosphate (pH 6.8), 0.087 M sodium chloride and 0.005 M magnesium chloride (Haugaard and Marsh, 1952). The final concentration of glucose was 0.4%. The insulin used was free of hyperglycemic factor and was added to a final concentration of 1 unit per milliliter. Four tenths ml. of 5 N H_2SO_4 was added to the center well and 0.4 ml. of 0.5 N NaOH to the side arm of each vessel. The alkali was prepared before each experiment from a saturated solution of NaOH and distilled water which had been boiled to remove carbon dioxide.

Oxygen uptake and the R.Q. were determined by a modification of the first method of Dickens and Simer (Umbreit *et al.*, 1949). Vessels were equilibrated for 15 minutes in the Warburg constant temperature bath at 37° C. during 5–6 minutes of which time they were gassed with 100% oxygen. At the beginning of the 90 minute experimental period during which respiration was measured, acid was tipped into the alkali and medium of blank vessels which contained no tissue in order to determine the initial CO_2 content of the medium and alkali. In the vessels containing tissue, carbon dioxide was being absorbed by the alkali and hence readings of the oxygen consumption could be made directly. At the end of 90 minutes incubation acid was tipped into the experimental vessels to allow for release of CO_2 for determination of this gas.

TABLE 1. EFFECT OF CORTISONE PRETREATMENT ON THE RESPIRATION OF BROWN AND WHITE FAT INCUBATED WITH AND WITHOUT GLUCOSE

	N*	No glucose	Glucose	Difference in paired vessels	P†
Brown fat-O_2 uptake			*μL./100 wet mgm./90 min.*		
Untreated rats	22	62.9 ±5.92†	61.6±5.32	−1.3 ±5.56	N.S.
Cortisone-treated rats	24	62.7 ±6.41	58.1±5.63	−4.6 ±4.25	N.S.
P‡		N.S.	N.S.		
White fat-O_2 uptake			*μL./100 wet mgm./90 min.*		
Untreated rats	18	7.3 ±0.63	8.5±0.97	+1.2 ±0.52	<.05
Cortisone-treated rats	22	6.3 ±0.52	6.6±0.74	+0.3 ±0.52	N.S.
P‡		N.S.	N.S.		
Brown fat—R.Q.					
Untreated rats	19	0.73±0.034	0.84±0.037	+0.11±0.027	<.01
Cortisone-treated rats	21	0.60±0.027	0.69±0.032	+0.09±0.035	<.02
P‡		<.01	<.01		
White fat—R.Q.					
Untreated rats	20	1.02±0.044	1.34±0.102	+0.32±0.085	<.01
Cortisone-treated rats	20	0.95±0.063	1.09±0.056	+0.14±0.083	N.S.
P‡		N.S.	<.05		

* N in this and the following table refers to the number of animals.
† All values expressed as Mean ±S.E.
‡ For both tables P in the horizontal columns represents results of statistical analysis between mean values for treated and untreated rats; P in vertical column refers to analyses of the mean difference between paired vessels.

RESULTS

The findings are summarized in Tables 1 and 2. It will be noted that a considerable difference in the respiratory activity of brown and white fat was demonstrable. Under all conditions studied the oxygen consumption of brown adipose tissue was higher and the R.Q. lower than in white fat.

TABLE 2. EFFECT OF INSULIN *in vitro* ON THE RESPIRATION OF BROWN AND WHITE FAT FROM UNTREATED AND CORTISONE TREATED RATS

	N	Glucose	Glucose + insulin	Difference in paired vessels	P
Brown fat-O_2 uptake		*μL./100 wet mgm./90 min.*			
Untreated rats	22	60.2 ± 9.64	54.8 ± 8.91	−5.3 ± 3.60	N.S.
Cortisone-treated rats	16	61.5 ± 9.79	63.8 ± 7.65	+2.3 ± 5.74	N.S.
P		N.S.	N.S.		
White fat-O_2 uptake		*μL./100 wet mgm./90 min.*			
Untreated rats	13	5.7 ± 0.63	6.6 ± 0.65	+0.9 ± 0.58	N.S.
Cortisone-treated rats	15	7.2 ± 0.76	7.6 ± 0.89	+0.4 ± 0.69	N.S.
P		N.S.	N.S.		
Brown fat—R.Q.					
Untreated rats	16	0.65 ± 0.033	0.68 ± 0.039	+0.03 ± 0.028	N.S.
Cortisone-treated rats	12	0.70 ± 0.032	0.68 ± 0.035	−0.02 ± 0.058	N.S.
P		N.S.	N.S.		
White fat—R.Q.					
Untreated rats	11	1.24 ± 0.099	1.26 ± 0.120	+0.02 ± 0.105	N.S.
Cortisone-treated rats	13	0.95 ± 0.044	0.95 ± 0.041	0 ± 0.046	N.S.
P		< .02	< .02		

None of the experimental procedures studied had any measurable effect on the oxygen consumption of either type of tissue but significant alterations in the R.Q. were recorded. Noteworthy was the effect of addition of glucose to the phosphate-saline medium, with consequent significant increase in the R.Q. of both brown and white fat (Table 1). Insulin, on the other hand, had no influence on the R.Q. of either tissue when tested in the presence of glucose in the medium (Table 2).

Cortisone pretreatment of the rats for 48 hours had a distinct influence on the R.Q. values in most experiments but not on the oxygen consumption. Thus, the R.Q. of brown fat both in the presence and absence of glucose in the medium was significantly lower in the cortisone-treated rats in one experiment (Table 1), although in the second experiment performed at a different time, no difference could be discerned in a glucose medium with or without added insulin (Table 2). In a glucose medium, on the other hand, the R.Q. of white adipose tissue from cortisone-treated rats was always significantly lower than that of the controls, even when insulin was added to the glucose medium. In the absence of glucose from the medium there was no difference in the R.Q. from control and cortisone-treated

rats (Tables 1 and 2). When paired tissues from cortisone-treated rats were incubated with and without glucose, a significant increase in the R.Q. was found with brown fat but not with white fat in contrast to the rather marked increase in the R.Q. of the white adipose tissue from control rats.

DISCUSSION

The results of these experiments are not entirely reconcilable with those of previous studies in the intact animal, although it must be noted that there were certain important differences in the design of the two experiments. In the present study, rats were fasted for 24 hours whereas in most of the earlier work 48-hour fasts were used. In the previous work measurements were made 2 to 10 hours after parenteral administration of glucose and insulin whereas in this investigation the glucose and insulin were added directly to the medium and effects measured for 90 minutes.

The increase in R.Q. when glucose was added to the medium and the lack of effect of insulin on the R.Q. confirm the results of Haugaard and Marsh (1952). This contrasts with the effects of insulin and glucose on glycogen levels in adipose tissue *in vivo* where insulin treatment caused a marked stimulation in glycogen accumulation. The lack of effect of insulin on the R.Q. is not consistent with the interpretation that the increase in glycogen reflects an increased rate of lipogenesis unless one assumes that there is a significant lag between glycogen deposition and fat formation. Wertheimer and Shapiro (1948) have suggested this. It may be significant in this regard that the lower R.Q. and smaller increment in R.Q. with glucose were noted in the brown fat which shows the greater changes in glycogen. The depression in R.Q. noted in most experiments in the cortisone-treated rats could not be correlated with any changes in the glycogen content of the adipose tissue of the intact rats. It will be recalled that cortisone had no effect on adipose tissue glycogen in glucose-fed rats but markedly increased it in insulin-treated glucose-fed rats (Engel, 1951, Engel and Scott, 1951). The lowering of the R.Q. by cortisone treatment, however, is consistent with the interpretation that this hormone inhibits the conversion of carbohydrate to fat.

The respiratory activity of brown fat was significantly higher than that of white fat, confirming the results of previous investigators (Mirski, 1942, Hook and Barron, 1941). However, the oxygen consumption of white fat in the present study was only about one-third that reported by Haugaard and Marsh (1952) using a comparable technique, although it was comparable to that reported by Henle and Szpingier (1936). Furthermore, no significant increase in oxygen consumption occurred on incubation of white fat with insulin. There is no obvious explanation for this discrepancy between our results and those of Haugaard and Marsh.

SUMMARY

The oxygen consumption and R.Q. of brown and white adipose tissue of 24-hour fasted normal and cortisone-treated rats have been measured *in vitro* with and without glucose and insulin.

The oxygen consumption of brown fat was significantly higher than that of white adipose tissue. The oxygen consumption was not modified by any experimental condition studied.

The R.Q. of white adipose tissue was significantly higher than that of brown adipose tissue.

The R.Q. of adipose tissue was increased by incubation with glucose, but not modified by insulin.

In various experiments pretreatment with cortisone for 48 hours either induced no change or a decrease in the R.Q. of adipose tissue incubated in medium alone, in glucose medium and in glucose and insulin medium. With white adipose tissue the increase in R.Q. with glucose was prevented by cortisone pretreatment.

These results are consistent with the interpretation that cortisone inhibits lipogenesis from carbohydrate but offer no basis to interpret the previously reported effects of insulin and cortisone on adipose tissue glycogen levels.

Acknowledgment

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A COMPARISON OF MYOTROPHIC AND ANDROGENIC ACTIVITIES OF TESTOSTERONE PROPIONATE WITH 19-NORTESTOSTERONE AND ITS ESTERS[1]

LESTER E. BARNES, ROBERT O. STAFFORD, MARILYN E. GUILD, LOU C. THOLE AND KENNETH J. OLSON

Research Laboratories, The Upjohn Company, Kalamazoo, Michigan

INTRODUCTION

IT HAS been established that certain androgenic steroids have metabolic effects other than the well-known trophic effects on the reproductive system. An androgen-treated animal frequently exhibits, in addition to the classical changes in the reproductive tract, other important changes which are associated with protein anabolism.

This effect is manifested in an increase in general body weight, increased kidney weight, and decreased urinary nitrogen excretion (Kochakian and Murlin, 1935; Kenyon, Knowlton, and Sandiford, 1944; McCullagh and Rossmiller, 1941; Kochakian, 1944, 1946, 1950; Kassenaar, van Bekkum, and Querido, 1953).

Another index of protein anabolism is the myotrophic response, or increases in size of skeletal muscles such as the temporal (Papanicolaou and Falk, 1938) or levator ani (Eisenberg and Gordan, 1950). The levator ani musele is comparatively easy to dissect, gives consistent responses on repeated assays, and appears not to be unduly influenced as a secondary sex characteristic by androgenic stimulation.

The levator ani method of Eisenberg and Gordan has been used in our laboratory to explore the myotrophic activity of a number of steroids. This communication reports the results of testing of the levator ani stimulating activity and seminal vesicle stimulating activity of 19-nortestosterone (Birch, 1950) and some of its esters.

Hershberger, Shipley and Meyer (1953) have also studied the androgenic and levator ani stimulating activity of a series of steroids including 19-nortestosterone.

METHODS

Weanling male rats of the Upjohn colony (Sprague-Dawley strain) weighing between

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[1] A paper describing the preparation and physical and chemical properties of these compounds is in press (J. Am. Chem. Soc.). Babcock, J. C., R. A. Donia, B. A. Johnson and A. C. Ott.

40 and 45 gm. were castrated and fed *ad libitum* with stock laboratory diet.[2] Beginning on the day following castration and for twenty days thereafter, the rats received a single daily subcutaneous injection of the test compound dissolved in 0.1 cc. cottonseed oil, five animals comprising a dosage group in any single experiment. On the day following the last injection, the rats were killed by chloroform and autopsied. Body weights were recorded. The seminal vesicles were used as an index of androgenic potency and the levator ani muscles were used to indicate myotrophic activity. Kidneys were removed and weighed as another index of anabolic activity (renotrophic effect). Dissection of the levator ani muscles followed the method of Eisenberg and Gordan (1950). Seminal vesicles were removed by cutting through the common duct. They were stripped free of adherent tissue, the coagulating glands were removed, any fluid was extruded, and the remaining tissue was blotted on paper toweling. Seminal vesicles and levator ani muscles were weighed to the nearest 0.1 mg. on a Roller-Smith torsion balance. The kidney capsules, renal blood vessels, ureters and adherent fat were removed, and the kidneys were weighed in pairs to the nearest 1.0 mg.

The organ weights were tabulated on the basis of milligrams per 100 gm. body weight. Testosterone propionate (TP) was run in parallel on all tests and the data from the entire series were analyzed by the method of Irwin (1937) in order that the potencies of every experimental compound could be compared, on the basis of seminal vesicle weights and levator ani weights, with the androgenic and myotrophic potencies of TP. Since it was not only the myotrophic potency but the ratio of myotrophic:androgenic potencies which were of interest, such a ratio for all compounds tested was obtained by the following calculation (see Table 4).

$$\frac{\dfrac{\text{Myotrophic potency of test compound}}{\text{Myotrophic potency of TP}}}{\dfrac{\text{Androgenic potency of test compound}}{\text{Androgenic potency of TP}}} = \text{Myotrophic:androgenic ratio of test compound}$$

RESULTS AND DISCUSSION

Levator Ani and Seminal Vesicle Weights (Myotrophic and Androgenic Effects)

19-nortestosterone. Table 1 and Figures 1 and 2 present the data from a series of tests comparing free 19-nortestosterone and TP. The preferential myotrophic activity of this steroid is demonstrated at all doses tested. Even at the highest dose of 500 μg. per day, the androgenic activity approximates that obtained with only 25 μg. of TP, whereas the myotrophic activity more nearly approximates that obtained at the 250 μg. dose of TP. These observations are expressed as calculated ratios of potencies in Table 5.

19-nortestosterone 17β-trimethylacetae. The data obtained with this derivative are presented in Table 2 and Figures 1 and 2. This ester demonstrated extremely low androgenic effects. At the highest dose used, 500 μg. per

[2] Archer-Daniels-Midland Company, Minneapolis, Minnesota.

Minimum Crude Protein	25%
Minimum Crude Fat	4%

TABLE 1. COMPARISON OF ACTIVITIES OF 19-NORTESTOSTERONE AND TESTOSTERONE PROPIONATE ON THE SEMINAL VESICLES AND LEVATOR ANI MUSCLES OF CASTRATE, WEANLING MALE RATS

Treatment	Daily dose (μg.)	No. of animals	Body weight	Organ weights[1]		
				Seminal vesicles	Levator ani	Kidney
Oil	—	15	149	4.6	23.6	970
Testosterone propionate	10	10	154	11.2	26.5	1023
	25	10	153	38.8	38.4	1076
	50	10	166	76.9	49.8	1074
	100	5	162	125.0	74.4	1021
	250	5	171	196.5	82.7	1130
	500	5	166	199.0	89.6	1107
19-nortestosterone	10	5	152	4.6	33.1	960
	25	5	156	5.6	38.1	992
	50	5	161	7.1	44.6	1049
	100	10	162	9.9	59.1	1025
	250	10	160	30.1	70.4	1058
	500	10	156	42.9	81.7	1003

[1] Organ weights presented as milligrams per 100 grams body weight.

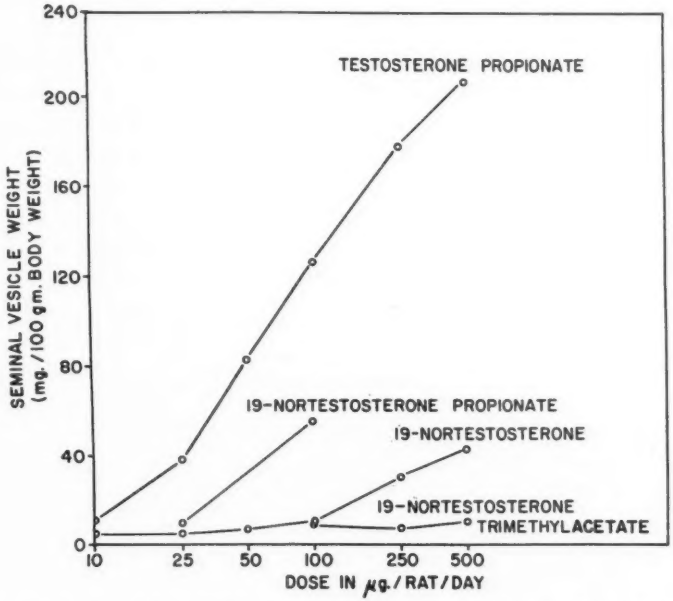

FIG. 1. Seminal vesicle weights of castrate, weanling male rats treated with testosterone propionate, 19-nortestosterone, and the trimethylacetate and propionate esters of 19-nortestosterone.

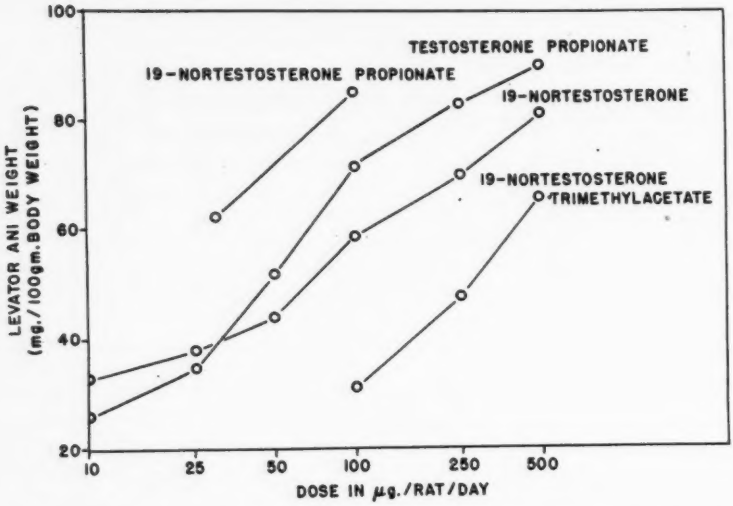

FIG. 2. Levator ani muscle weights of castrate, weanling male rats treated with testosterone propionate, 19-nortestosterone, and the trimethylacetate and propionate esters of 19-nortestosterone.

day, the androgenic response measured by seminal vesicle weight was only slightly higher than that obtained in the control groups, whereas the myotrophic activity was approximately 20% that of TP. While the myotrophic potency of this compound in comparison with the other esters tested in this series was relatively low, these factors combined to present a very large myotrophic:androgenic ratio (see Table 5).

19-nortestosterone 17β-propionate. Table 3 and Figures 1 and 2 summarize the data from one experiment comparing TP and 19-nortestosterone pro-

TABLE 2. COMPARISON OF ACTIVITIES OF 19-NORTESTOSTERONE TRIMETHYLACETATE AND TESTOSTERONE PROPIONATE ON THE SEMINAL VESICLES AND LEVATOR ANI MUSCLES OF CASTRATE, WEANLING MALE RATS

Treatment	Daily dose (µg.)	No. of animals	Body weight	Organ weights[1]		
				Seminal vesicle	Levator ani	Kidney
Oil	—	5	147	4.9	25.9	996
Testosterone propionate	100	5	169	116.5	71.6	1029
	250	5	170	161.3	84.8	1052
	500	5	165	204.7	94.0	1050
19-nortestosterone trimethylacetate	100	5	145	8.6	30.7	1080
	250	5	145	7.9	48.0	1022
	500	5	157	10.7	66.4	1051

[1] Organ weights presented as milligrams per 100 grams body weight.

pionate. At the two doses used, the propionate ester appeared to be a powerful myotrophic agent, displaying myotrophic activity superior to that of TP while retaining the quality of low androgenic activity in comparison to TP. These two factors produced a high myotrophic:androgenic ratio. Also, as seen in Table 5, its actual potency is very high; the myotrophic effect obtained with 1 mg. of TP could theoretically be duplicated with less than one-half the amount of this derivative.

TABLE 3. COMPARISON OF ACTIVITIES OF 19-NORTESTOSTERONE PROPIONATE AND TESTOSTERONE PROPIONATE ON THE SEMINAL VESICLES AND LEVATOR ANI MUSCLES OF CASTRATE, WEANLING MALE RATS

Treatment	Daily dose (μg.)	No. of animals	Body weight	Organ weights[1]		
				Seminal vesicle	Levator ani	Kidney
Oil	—	5	141	5.4	21.9	1022
Testosterone propionate	25	5	149	32.9	30.7	1071
	100	5	162	142.0	70.2	1168
19-nortestosterone propionate	25	5	162	9.1	62.2	1067
	100	5	160	55.1	85.6	1168

[1] Organ weights presented as milligrams per 100 grams body weight.

19-nortestosterone 17β-cyclopentylpropionate. Table 4 and Figures 3 and 4 show the data from a number of tests comparing this compound to TP. The myotrophic activity of 19-nortestosterone cyclopentylpropionate was superior to that of TP at all dose levels tested, and the androgenic activity

TABLE 4. COMPARISON OF ACTIVITIES OF 19-NORTESTOSTERONE CYCLOPENTYLPROPIONATE AND TESTOSTERONE PROPIONATE ON THE SEMINAL VESICLES AND LEVATOR ANI MUSCLES OF CASTRATE, WEANLING MALE RATS

Treatment	Daily dose (μg.)	No. of animals	Body weight	Organ weights[1]		
				Seminal vesicle	Levator ani	Kidney
Oil	—	49	154	4.3	25.1	1009
Testosterone propionate	25	20	161	39.9	36.1	1025
	50	20	160	89.7	54.0	1060
	100	23	170	122.8	70.7	1030
	250	20	172	177.7	81.7	1025
	500	5	161	219.6	87.2	1080
19-nortestosterone cyclopentylpropionate	12.5	15	157	5.9	35.7	998
	25	19	155	6.4	51.7	1058
	50	20	164	9.1	65.8	1038
	100	10	156	24.4	84.6	1179
	125	14	160	24.0	78.9	1071
	250	5	166	78.9	87.8	1111
	500	5	164	124.1	97.6	1192

[1] Organ weights presented as milligrams per 100 grams body weight.

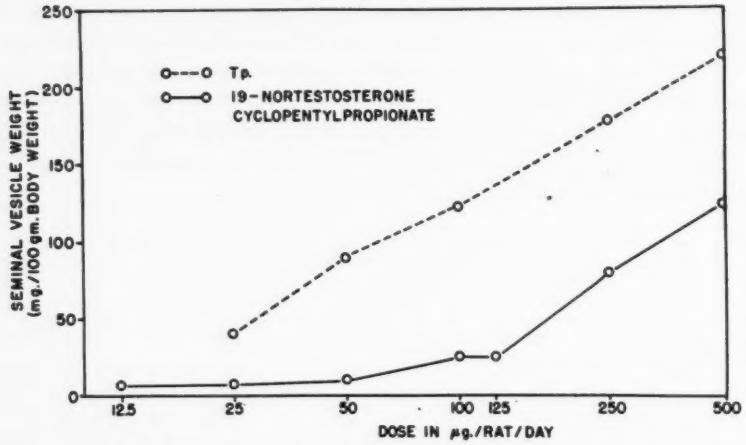

FIG. 3. Comparison of seminal vesicle weights of castrate, weanling male rats treated with testosterone propionate and 19-nortestosterone cyclopentylpropionate.

was negligible until levels of 100 µg. or more per day were administered. At the highest dose tested, the androgenic activity approximated that obtained with one-fifth the dose of TP; on the other hand, at the low daily dose of 12.5 µg., the myotrophic activity was equal to that obtained with approximately twice the dose of TP.

This ester exhibits the potent myotrophic property of the propionate ester with only about one-half as much androgenic activity. Thus, of the five compounds tested in this study, this compound has the greatest myotrophic potency per unit weight with the least relative androgenic activity.

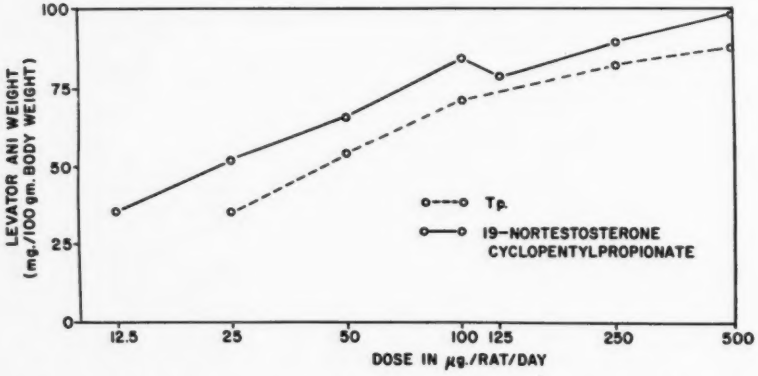

FIG. 4. Comparison of levator ani muscle weights of castrate, weanling male rats treated with testosterone propionate and 19-nortestosterone cyclopentylpropionate.

TABLE 5. SUMMARY OF POTENCIES OF 19-NORTESTOSTERONE AND ITS ESTERS COMPARED WITH TESTOSTERONE PROPIONATE

Test compound	Potency ratio of test compound to TP		Ratio of myotrophic to androgenic potency	Am't required to produce myotrophic effect of 1 mg. TP
	"Androgenic" activity	"Myotrophic" activity		
Testosterone Propionate[1]	1.00	1.00	1.0	1.0
19-nortestosterone	.07	.66	9.4	1.5
19-nortesterone trimethylacetate	<.02	.18	>9.0	5.5
19-nortestosterone propionate	.41	2.59	6.3	0.4
19-nortestosterone cyclopentylpropionate	.21	2.02	9.6	0.5

[1] For the purpose of this tabulation, testosterone propionate as the standard androgen is given a myotrophic: androgen ratio of 1.0.

Kidney Weights (Renotrophic Effect)

The data on kidney weights throughout the experiment did not lend themselves well to statistical analysis or thorough interpretation. Small, inconsistent increases in kidney weight were caused by most of the compounds tested. These results are suggestive of those reported by others (Ludden, Krueger, and Wright, 1941; Korenchevsky, Dennison, and Eldridge, 1937) for short-term experiments. Kochakian, Bartlett, and Trybalski (1946) have reported that it is necessary to administer androgens for 100 days or more before significant kidney weight increases are obtained in the rat.

The kidney weights are included in this report as a secondary indication of protein anabolic effects since, particularly in the TP and 19-nortestosterone cyclopentylpropionate data, definite trends toward increased kidney weight with increased dosage are indicated.

SUMMARY AND CONCLUSIONS

Investigation of the preferential myotrophic activity of oil solutions of 19-nortestosterone and three esters of 19-nortestosterone on the basis of levator ani weight and seminal vesicle weight has been reported.

19-nortestosterone was found to have more than one-half the myotrophic potency of testosterone propionate and less than one-tenth the androgenic potency.

The trimethylacetate ester of 19-nortestosterone exhibited very low androgenic activity at all doses tested and relatively high myotrophic activity.

The propionate ester exerts a myotrophic activity more than twice that obtained with TP and has an androgenic potency less than one-half that of TP.

The cyclopentylpropionate ester of 19-nortestosterone has a myotrophic

action approximately twice that of TP, but only about one-fifth the androgenic properties of TP, factors which produce a high myotrophic:androgenic ratio and high absolute potency.

Acknowledgment

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THE ACTION OF SELECTED DRUGS ON DECIDUOMA FORMATION

M. C. SHELESNYAK

Department of Experimental Biology, Weizmann Institute of Science, Rehovot, Israel

INTRODUCTION

RECENT attempts to explore the mechanism of blastocyst implantation were based on the assumption that the study of specific local factors which initiated the formation of the deciduoma (Loeb, 1907) might throw light on the natural nidation mechanism. Since the instillation of antihistaminic drugs into the uterine lumen of pseudopregnant rats at the time of endometrial traumatization inhibited the development of deciduomal tissue, it was assumed that histamine plays a role in the initiation of deciduomata (Shelesnyak, 1952, b, and 1953, 1954). Histamine effects are, however, multiple, and show species differences; and various antihistamines show differential suppression of the histamine actions. A study of the comparative effectiveness of various antihistamines on the suppression of the deciduoma formation revealed that antihistamines studied were effective, but to different degrees; and, moreover, that there was no correlation between rank order effectiveness of antihistamine deciduoma-inhibition and the ancillary action of the drugs (Shelesnyak, 1954).

In discussing specificity of antihistamine drugs Loew (1950) stated: "When antihistamine drugs, even though relatively specific, are used as research tools or diagnostic aids, due consideration must be given to a variety of pharmacological actions when experiments are planned and the findings interpreted."

This is a report of investigations planned to focus the mechanism of the antihistaminic drug inhibition of the artificial decidual cell reaction by the employment of various drugs chosen to emphasize the ancillary actions of the histamine antagonists. These include: local anesthetics, vasopressins, oxytocins, atropine and epinephrine.

METHODS AND MATERIALS

Adult female albino rats weighing from 150 to 200 grams were used. The animals were housed in air-conditioned quarters and fed Ralston-Purina chow with weekly

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[1] The author wishes to express his thanks to Dr. R. K. Richards, Abbott Laboratories, for the supply of Serotonine creatinine.

supplements of seasonal greens. Fresh drinking water was continually available. Vaginal smears were taken from all animals daily, during the first hours of the working day (between 0700 and 0930). After cyclic regularity was recorded, pseudopregnancy was provoked by means of electrical stimulation (Shelesnyak, 1931). On the fourth day of leucocyte smear, that is, the fifth day after electrical stimulation at proestrus, the animals were anesthetized with ether and both horns of the uterus exposed through a small mid-ventral abdominal incision. Uterine mucosa of both horns was traumatized with an 18 gauge hypodermic needle, inserted into the lumen near the bifurcation above the cervix and passed up toward the uterotubal junction, then withdrawn at an angle so that the antemesometrial wall was scratched.

In 125 cases, drugs were administered directly into the lumen of the uterus; in 52 animals by subcutaneous injection. In cases where drugs were applied directly into the uterus, one horn (usually the right) was instilled with 0.1 ml. Ringer's solution containing the drug under study by use of a 27 gauge $\frac{1}{2}''$ hypodermic needle, inserted just above the point of entry of the traumatizing needle. The contralateral horn was instilled with Ringer's fluid or was untreated except for traumatization.

The second group of 52 animals received drugs (except local anesthetics) by subcutaneous route at the time of uterine traumatization.

The following nine drugs were used: tetracaine dibucaine and chlorbutanol (local anesthetics); pitressin and serotonine creatinine (vasopressors); ergometrine maleate and pitocin (oxytocins); and atropine sulfate and epinephrine tartrate. The table shows the number of cases and treatment.

On the fourth day after traumatization and drug application the animals were autopsied. Whole uteri were mounted on cards and fixed in Bouin's solution or neutral formalin fixative. Photo silhouettes were made to record size and shape to allow for gross comparison of both horns and of different animals. Random samples, selected for microscopic study, were sectioned at 6 microns and stained with Harris haematoxylin and eosin.

RESULTS

Epinephrine tartrate was the most effective inhibitor of the decidual growth. One-hundredth to 0.13 mgm. *in utero* suppressed the development of the deciduoma. Oxytocins instilled into the uterine lumen also inhibited the decidual cell response to trauma, as did large doses of atropine sulfate. The local anesthetics (chlorbutanol, tetracaine and dibucaine) were ineffective in suppressing the uterine reaction when given in normally employed doses, as were both pitressin and serotonine creatinine. None of the drugs in this study was effective in suppressing the decidual reaction when administered subcutaneoulsy (see Table).

Random microscopic sections revealed a normal diestral histology in the inhibited horns when suppression was complete, or nests of decidual cells or small deciduomata depending upon the degrees of suppression. In some cases where atropine sulfate was instilled into the uterine lumen in doses of 5 mg. and greater, haemorrhagic necrosis was seen in the constricted uterus.

DISCUSSION

The suppression of the growth and development of deciduomata can be

INFLUENCE OF DRUGS ON DECIDUOMA FORMATION

Treatment			Number of cases	Results	Remarks
Drug	Dosage, mg. per 0.1 ml.	Route		Deciduomal suppression experimental horn*	
Chlorbutanol	0.5	Intra-uterine	6	0	
Dibucaine (Nupercaine)	1.0	Intra-uterine	6	0	
Tetracaine (Pantocain)	1.0 2.0	Intra-uterine	6 3	0 0→±	Dose level toxic
Beta-hypophamine (Pitressin)	0.5 P.U. 1.0 P.U. 2.0 P.U.	Intra-uterine	3 3 3	0 0 0	Dosage in pressor units
	5.0 P.U. 10.0 P.U. 20.0 P.U.	Subcutaneous	2 2 2	0 0 2	
Serotonine creatinine	1.0	Intra-uterine	6	0	
	10.0	Subcutaneous	6	0	
Pitocin	1 I.U.	Intra-uterine	12	+++→++++	Dosage in international units
	2 I.U. 8 I.U.	Subcutaneous	6 6	0 0	
Ergometrine maleate	0.05 0.1 0.25 0.5 1.0	Intra-uterine	4 1 2 3 4	0 0 ±→++ ++→++++ ++++	
	2.0 4.0 5.0 10.0	Subcutaneous	2 2 2 2	0 0 0 0	
Atropine sulfate	1.0 1.6 2.5 5.0 10.0 25.0 50.0	Intra-uterine	8 2 2 6 6 6 6	0 0 0 0→++ ±→++ +→++++ ++→++++	Haemorrhagic necrosis common in suppressed horns
	25.0 50.0 75.0	Subcutaneous	5 5 3	0 0 0	
Epinephrine tartrate	0.0018 0.018 0.09 0.135 0.18 0.36 0.45	Intra-uterine	1 2 3 6 6 5 4	0 0 ±→+++ ++++ ++++ ++++ ++++	Some suppression in control horn Some suppression in control horn Some suppression in control horn Complete bilateral suppression
	0.45 0.7 0.9	Subcutaneous	3 3 1	0 0 0	

* Key:
0—No suppression—extensive deciduomata.
±—Slight suppression in mid-section of horn.
+—Slight suppression.
++—Moderate suppression with slight suppression at tubal end.
+++—Marked suppression.
++++—Complete suppression along entire horn of uterus.

achieved by two different methods, each based on one of the two requirements for the formation of the deciduoma; namely, by interfering with (1) the proper hormonal conditioning of the uterus or (2) the stimulation of the endometrium. The first method has been explored by Courrier (1930) Rothschild, Meyer, and Spielman (1940), Hisaw and Velardo (1951) and Velardo and Hisaw (1951). These experiments involved the disturbance

of the endocrine status of the uterus by administration of estrogens and various steroids, and have been summarized by Courrier (1950).

The second method employs the intra-uterine application of drugs, and apparently operates at a cellular level, interfering with the stimuli required to initiate the deciduomal formation (Shelesnyak). These investigations aim to elicit some metabolic inciter of the progravid endometrium which provokes decidual cell formation. They suggested a relationship between histamine and deciduoma development on the basis of the specific inhibition by histamine antagonists topically applied to the site of endometrial traumatization.

The experiments reported here sharpen the focus on the mechanism but do not clearly define the agent or agents involved. Chambon and Lefrein (1952) suggested a vascular mechanism as the basis for the decidual response in the rabbit to histamine. However, the vasopressors other than epinephrine tartrate used in these studies did not inhibit decidual development. The suppressive action of the oxytocic drugs, pitocin and ergometrine maleate is consistent with histamine antagonism, since in the rat the response to histamine is uniquely the reverse of that in all other species of animals studied, and consists of uterine relaxation instead of the common contraction. The suppressive action of epinephrine tartrate is within expectations if the supposition is made that histamine initiates the decidual reaction. Since the effective inhibitory dose of epinephrine is very small, and since large doses of atropine sulfate suppress deciduomal formation, the possibility presents itself that the mechanism of decidual cell initiation may involve the triad of adrenergic-cholinergic-histaminic systems. Evidence has not been found which alters the suggestion that histamine is involved in the initiation of the deciduomata (Shelesnyak, 1952b).

SUMMARY

Various drugs, selected for activities similar to the ancillary effects of antihistamines, were investigated in regard to suppression of deciduomata formation in order to focus the physiological factors.

Epinephrine tartrate was very effective, pitocin and ergometrine maleate were less so, and atropine in large doses, was effective in suppressing the development of deciduomata when instilled *in utero* at the time of endometrial traumatization. Pitressin, serotonine, creatinine and tetracaine, chlorbutanol and dibucaine failed to interfere with deciduoma formation.

From these studies local anesthetic and vasopressin effects may be eliminated as the factor of deciduomal inhibition. The role of histamine as an incitor is substantiated, although the action of epinephrine and large

doses of atropine as deciduoma inhibitors suggest the involvement of the triad of adrenergic-cholinergic-histaminic system.

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THE LYMPHOCYTOTIC RESPONSE AS AN INDICATOR OF RELATIVE ADRENOCORTICAL INSUFFICIENCY[1]

L. F. KUMAGAI AND T. F. DOUGHERTY

Department of Anatomy, University of Utah College of Medicine, Salt Lake City, Utah

INTRODUCTION

IT HAS been demonstrated that stress stimuli induce a lymphocytic response which is not mediated by pituitary adrenocortical hormones. This non-adrenocortical response is characterized by a lymphocytosis which appears earlier and rises to higher levels than the lymphocytosis due to adrenalectomy alone (Dougherty and Kumagai, 1951). The number of lymphocytes of stressed adrenalectomized animals return to that level found in non-stressed adrenalectomized animals within 24 hours following the stress stimulus. These sequential changes in the number of circulating lymphocytes represent in their numercial and temporal characteristics an inverse image of the curve of the "lymphopenic response" and have been termed the "lymphocytotic response."

The lymphatic organs of stressed adrenalectomized animals also undergo a rapid increase in size. The rate of increase in amount of lymphatic tissue as measured by weight and nitrogen content exceed that found following adrenalectomy alone. It was suggested that stress enhances the rate of lymphocytopoiesis by augmenting the release of or action of as yet unknown lymphocyte growth promoting factors (Dougherty and Kumagai, 1951). This stress-induced augmentation of lymphocytopoiesis appears to be masked in the intact animals by the release of adrenocortical hormones which in turn exert lymphocytolytic and growth inhibiting influences on lymphatic tissue.

The following experiments were performed in order to provide a further understanding of the quantitative relationship existing between the non-adrenocortical mediated stress-induced lymphocytotic response and the pituitary adrenocortical mediated lymphopenic response.

MATERIALS AND METHODS

A total of 296 CBA mice of both sexes between 12 and 16 weeks of age were used in these studies. All animals were fed *ad libitum* on dog chow (Purina) supplemented with

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calf meal (Larro) before and during the experimental period. Animals were maintained in quarters kept at a constant temperature. Tail blood was used for all leucocyte counts and differential counts were performed on blood films stained with May-Grünwald Giemsa, at least 200 cells being differentially counted in each film. Bilateral adrenalectomy was performed by the usual dorsal approach.

Two groups of animals served as controls. The first group consisted of non-treated intact mice from which control values for total and differential leucocyte counts were established. The second control group was made up of non-stressed adrenalectomized mice of the same age and sex as those used in the stress experiments. Leucocyte values were obtained on these animals at the various time intervals after adrenalectomy as shown in Table 1.

TABLE 1. HAEMATOLOGICAL VALUES OF CONTROL INTACT, CONTROL ADRENALECTOMIZED AND ADRENALECTOMIZED MICE MAINTAINED ON CORTISONE

Groups	Number of animals	Time after adrenalectomy	W.B.C. thousands per cmm.	Polymorphonuclear thousands per cmm.	Lymphocytes thousands per cmm.	Eosinophils per cmm.
			Mean S.E.	Mean S.E.	Mean S.E.	Mean S.E.
Intact controls*	150	—	12.58±0.27	3.87±0.12	8.60±0.23	117± 8
Adrenalectomized* controls	12	4 hrs.	15.22±1.32	4.54±0.57	10.62±0.96	55±25
	14	8 hrs.	11.00±0.90	2.77±0.12	8.18±0.71	51±20
	13	12 hrs.	12.85±0.60	4.38±0.48	8.39±0.52	67±25
	16	24 hrs.	23.15±3.29	7.06±1.31	15.95±2.04	115±40
	10	2 days	17.98±1.55	3.43±0.93	14.49±1.14	63±27
	10	4 days	19.28±1.48	3.78±0.46	15.27±1.42	242±69
	10	6 days	22.04±1.21	7.12±0.78	14.56±0.87	357±52
Adrenalectomized +0.5 mg. cortisone acetate daily	10	2 days	8.47±0.80	1.72±0.25	6.70±0.70	30±16
	9	4 days	7.57±1.03	3.49±0.46	4.05±0.73	27±13
	10	6 days	5.65±1.15	2.52±0.32	3.10±0.85	0
	8	8 days	11.45±1.70	6.35±0.75	4.95±1.15	43±22
Adrenalectomized +0.05 mg. cortisone acetate daily	13	2 days	16.59±1.32	5.04±0.59	11.27±1.08	281±62
	12	4 days	15.60±1.21	4.53±0.38	10.88±1.18	182±57
	11	6 days	16.11±1.33	4.86±0.43	10.78±1.01	370±83
	12	8 days	11.01±0.85	3.01±0.37	7.88±0.76	118±28
Adrenalectomized +0.005 mg. cortisone acetate daily	8	2 days	13.49±1.17	5.54±0.67	7.84±0.77	192±60
	8	4 days	11.64±0.86	4.22±0.53	7.27±1.02	176±46
	8	6 days	22.24±1.48	3.96±0.28	18.09±0.59	192±55
	8	8 days	21.71±2.39	3.93±0.52	17.57±2.09	517±13

* Control data published previously (Dougherty, T. F. and Kumagai, L. F. *Endocrinology* **48**: 691, 1951) with the exception of the eight hour adrenalectomized group. These control values were obtained at approximately the same time as the experimental data, and are included here so that comparison between the effects of adrenalectomy alone and treatment of adrenalectomized animals with graded doses of cortisone can be made.

The experimental animals consisted of adrenalectomized mice which were maintained for eight days on graded doses of cortisone acetate. These animals were adrenalectomized 8 hours prior to the first injection of hormone and were divided into 3 groups, each of which were given intraperitoneal injections of cortisone daily (0.5 mg., 0.05 mg. and 0.005 mg.), as indicated in Table 1. The total daily dose of hormone was divided into 3 equal amounts and was injected at 8-hour intervals for the maintenance period. Blood counts were obtained immediately prior to the morning injection of hormone at 2-day intervals during the period of treatment. Immediately after the last hormone injection on the 8th day, all animals were given a single subcutaneous dose of 750 mg./kg. of histamine diphosphate. No further hormone was given after the histamine was administered. The animals surviving this dose were then given successive subcutaneous injections of 750 mg./kg. of histamine diphosphate at 2-day intervals. Blood counts were obtained after each stress stimulus at the time intervals as shown in Figure 1. The per cent of mortality following each stress stimulus is shown in Figure 2.

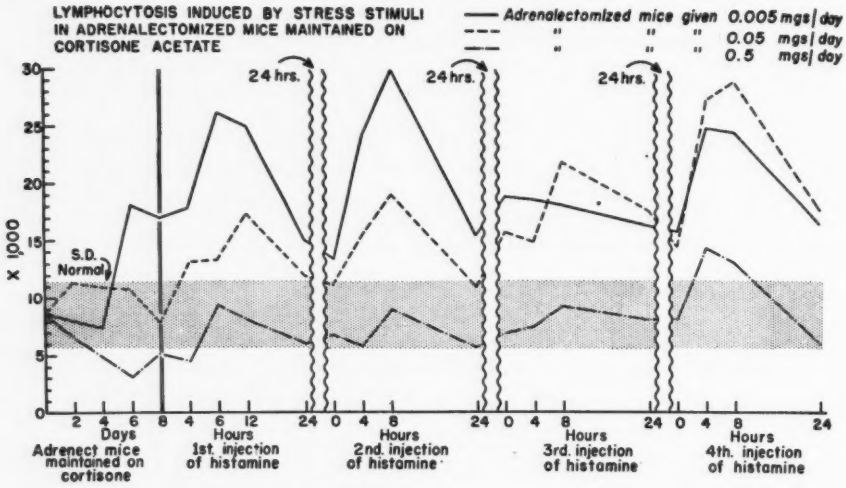

FIG. 1. Absolute numbers of circulating lymphocytes of adrenalectomized mice pretreated with graded amounts of cortisone acetate daily and then subjected to standard stress stimuli (histamine diphosphate at a dose of 750 mg./kgm. given subcutaneously at 48 hour intervals). The stippled area represents the standard deviation from the mean number of lymphocytes observed in non-stressed intact mice.

RESULTS

Alterations in the leukocytes of non-stressed mice following adrenalectomy. The changes in hematologic values following adrenalectomy have been described in previous publications (Dougherty and Dougherty, 1950; Dougherty and Kumagai, 1951). It may again be pointed out that the lymphocytosis characteristically found in adrenalectomized mice does not occur until 24 hours after operation (Table 1).

Effect of daily administration of various amounts of cortisone on leucocytes of adrenalectomized mice. As demonstrated in Tables 1 and 2 (zero hour value before first histamine injection), daily injections of 0.5 mg. cortisone acetate maintained a lymphopenia in adrenalectomized mice for the 8-day period of study. Daily administrations of 0.005 mg. cortisone acetate did not prevent a lymphocytosis during the maintenance period. The intermediate dose of 0.05 mg. cortisone acetate given daily maintained the hematologic values within normal limits during the entire 8-day period of study. This amount of hormone is referred to as the "Effective lymphocyte maintenance dose."

Effect of successive dosages of histamine on leucocyte values of adrenalectomized mice pretreated with cortisone. After the initial administration of

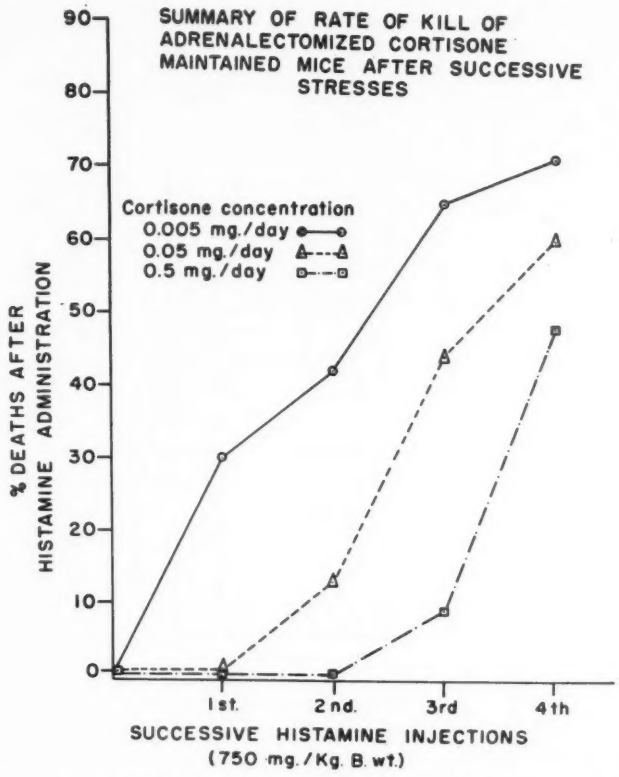

FIG. 2. Comparison of the per cent mortality of adrenalectomized mice pretreated with graded amounts of cortisone acetate daily, then subjected to successive standard stress stimuli at 48-hour intervals.

histamine the total leucocyte counts increased in the groups that had received 0.05 mg. and 0.005 mg. cortisone acetate daily during the maintenance period and remained relatively unchanged in the 0.5 mg. group (Table 2).

A rapid increase in the number of lymphocytes (Lymphocytotic Response) after administration of the stressor was observed in both the 0.05 mg. and 0.005 mg. groups. The response was significantly greater in the 0.005 mg. group of mice (Fig. 1). Although the numbers of lymphocytes in the 0.5 mg. group increased above their previous lymphopenic level, they did not go beyond the normal limits (Fig. 1). Within 24 hours after the initial injection of histamine, the lymphocyte values of all groups returned to approximately the same levels observed prior to the stress administration.

TABLE 2. HAEMATOLOGICAL VALUES OF SUCCESSIVELY STRESSED ADRENALECTOMIZED ANIMALS PREVIOUSLY MAINTAINED ON GRADED DOSES OF CORTISONE

Groups	Number of animals	Hours after stress	W.B.C., thousands per cmm.	Polymorphonuclears, thousands per cmm.	Lymphocytes, thousands per cmm.	Eosinophils per cmm.
			Mean S.E.	Mean S.E.	Mean S.E.	Mean S.E.
1st Histamine Injection						
750 mg./kg. S.C.						
0.5 mg.*	8	0†	11.45±1.70	6.35±0.75	4.95±1.15	43± 22
	12	4	14.88±1.25	10.60±1.00	4.30±0.60	0± 0
	12	8	15.05±1.55	5.70±0.45	9.15±1.35	80± 30
	11	12	15.35±1.70	6.50±0.85	8.85±1.30	40± 20
	12	24	11.05±1.00	5.05±0.50	5.95±0.75	50± 15
0.05 mg.*	12	0†	11.01±0.85	3.01±0.37	7.88±0.76	118± 28
	12	4	22.15±1.45	9.00±0.65	13.10±2.10	60± 25
	12	8	20.25±1.30	6.80±0.80	13.30±1.40	145± 25
	12	12	22.50±1.40	5.00±0.70	17.40±1.10	200± 70
	11	24	16.10±1.90	3.90±0.50	12.05±1.70	160± 45
0.005 mg.*	8	0†	21.71±2.39	3.93±0.52	17.57±2.09	517±131
	12	4	27.10±2.28	9.20±0.95	17.65±2.10	190± 65
	12	8	44.45±6.00	17.40±2.55	26.55±3.65	530±110
	12	12	39.80±8.08	14.45±3.35	25.00±4.95	440± 85
	11	24	20.50±2.95	5.10±0.85	15.10±2.10	215± 85
2nd Histamine Injection						
750 mg./kg. S.C.						
0.5 mg.	5	0	14.15±1.25	7.15±0.90	6.85±0.90	95± 60
	10	4	14.50±1.70	8.85±1.35	5.60±0.95	15± 15
	10	8	19.80±1.60	8.80±0.95	10.95±0.85	15± 15
	10	24	11.40±0.65	5.70±0.55	5.70±0.55	60± 20
0.05 mg.	5	0	17.45±2.20	5.85±0.70	11.40±2.25	200± 40
	10	4	26.65±3.30	9.50±1.55	16.30±2.10	130± 35
	10	8	27.20±2.55	8.00±1.15	19.10±1.65	110± 30
	10	24	17.30±1.65	5.95±1.10	11.15±0.85	135± 45
0.005 mg.	5	0	21.65±2.05	8.00±1.20	13.40±2.40	260± 70
	10	4	34.30±3.35	10.75±1.55	24.15±2.60	270± 75
	10	8	46.50±7.35	16.15±3.85	30.10±4.25	195± 60
	10	24	22.95±3.65	7.10±2.00	15.65±2.05	185± 60
3rd Histamine Injection						
750 mg./kg. S.C.						
0.5 mg.	5	0	12.95±1.00	5.85±0.75	7.05±0.95	40± 25
	10	4	17.85±2.40	10.35±1.50	7.45±1.15	50± 20
	10	8	16.60±2.15	7.15±1.55	9.40±1.45	70± 45
	10	24	11.40±0.80	3.65±0.50	7.65±0.75	95± 60
0.05 mg.	5	0	23.30±3.95	7.30±1.80	15.80±2.20	225±115
	10	4	27.30±1.85	12.35±1.90	14.90±0.90	70± 45
	10	8	35.15±5.45	12.95±2.30	22.50±4.35	125± 45
	10	24	22.25±3.45	4.50±0.85	17.55±2.60	140± 40
0.005 mg.	5	0	28.80±1.55	9.50±1.15	18.90±0.55	495±200
	10	4	28.55±3.20	9.85±1.30	18.70±2.25	110± 45
	10	8	29.10±1.94	10.60±1.60	18.30±1.15	160± 50
	10	24	21.10±2.30	4.35±0.55	16.55±2.10	185± 45
4th Histamine Injection						
750 mg./kg. S.C.						
0.5 mg.	5	0	14.05±1.50	6.20±1.10	7.65±0.45	200± 65
	10	4	24.55±4.30	9.90±1.75	14.50±3.25	150± 60
	10	8	23.80±1.50	10.20±1.95	13.30±3.00	315± 95
	10	24	11.20±1.25	4.55±0.50	6.40±0.90	200± 50
0.05 mg.	5	0	23.90±3.00	8.85±2.00	14.60±1.50	435± 60
	10	4	39.75±5.80	11.60±1.60	27.45±4.65	480±135
	10	8	39.70±5.00	10.85±2.15	29.10±3.95	470±130
	10	24	28.85±5.90	10.35±1.85	17.50±4.10	1050±260
0.005 mg.	5	0	25.30±3.20	9.15±2.65	15.85±1.95	265± 75
	10	4	34.00±5.00	8.90±1.55	25.00±3.85	115± 35
	10	8	34.70±4.15	9.65±2.00	24.15±2.70	500±130
	9	24	25.10±2.30	8.10±1.30	15.05±2.35	335±100

† Control values obtained on 8th day of maintenance period immediately before cortisone and histamine administration.

* 0.5 mg.: Adrenalectomized mice given 0.5 mg. of cortisone acetate daily for 8 days prior to stress.
0.05 mg.: Adrenalectomized mice given 0.05 mg. of cortisone acetate daily for 8 days prior to stress.
0.005 mg.: Adrenalectomized mice given 0.005 mg. of cortisone acetate daily for 8 days prior to stress.

The alterations in the number of polymorphonuclears and eosinophils were quite variable; however, they reflected similar directional changes as did the lymphocytes at comparable time intervals.

The lymphocytes in all three groups responded in essentially the same manner as described above following the second injection of histamine which was given 2 days after the initial administration (Fig. 1).

Following the third injection of histamine, it was noted that the difference in the degree in "lymphocytotic responses" observed previously in the 0.05 mg. and 0.005 mg. groups no longer existed. The alterations in leucocyte values were similar in all other respects to those seen following the first and second administrations of histamine.

The total leucocyte count was increased in all groups after the fourth administration of histamine (Table 2). Four hours after stress a significant lymphocytosis was observed for the first time in the group of animals that had been pretreated with 0.5 mg. cortisone acetate daily. As previously stated the lymphocytes in the animals of this group increased after each histamine injection; however, prior to the fourth injection, they did not increase beyond the normal limits as indicated in Figure 1. Typical lymphocytotic responses were also observed in the 0.05 mg. and 0.005 mg. groups; both groups responded to a much greater degree than did the 0.5 mg. animals. The polymorphonuclears remained fairly constant in all groups. A markedly significant eosinophilia was observed in the 0.05 mg. group during the entire 24-hour period of study following the fourth administration of histamine. The eosinophils in the 0.5 mg. and 0.005 mg. groups increased significantly at some time intervals as shown in Table 2. There did not appear to be as consistent a pattern for eosinophil response although in general they were similar to changes for lymphocytes.

Analysis of percentage mortality of adrenalectomized mice pretreated with graded doses of cortisone and subjected to successive histamine injections. As indicated in Figure 2, 30% of the group of mice that had received the least amount of hormone (0.005 mg. daily) during the maintenance period died after the first histamine injection. A comparable number of deaths were not observed in the 0.5 mg. group until after the fourth administration of histamine which was 8 days after hormone treatment was discontinued. The animals that had been previously maintained with 0.05 mg. of cortisone acetate daily showed intermediate results in the rate of kill following successive histamine injections.

DISCUSSION

It has been demonstrated previously that in the absence of the adrenal cortex, not only is the lymphopenia which follows stress stimuli abolished, but there is a significant lymphocytosis (lymphocytotic response) (Dough-

erty and Kumagai, 1951). The data presented indicate that the lymphocytotic response to stress occurs not only in the absence of the adrenal cortex, but may follow a stress stimulus when a relative adrenal cortical insufficiency exists, i.e., when the amount of hormone available is insufficient simultaneously to maintain lymphatic tissue and to meet the increased demands for utilization induced by stress. Conversely, when the amount of hormone available is present in excess concentration a lymphopenic response occurs rather than a lymphocytotic response. This type of phenomenon is usually observed as a physiological response of the intact organism to an acute stress when a sudden excessive amount of hormone is made available from adrenocortical hypersecretion.

It is possible, however, that under conditions of severe chronic stress (or multiple stresses) such as certain chronic diseases, e.g. tuberculosis or syphilis; or, in the presence of an acute overwhelming stress, the amount of available hormone may be insufficient to perform adequately its physiological role in the body and to meet simultaneously the increased demands induced by the stress. Such situations would be examples of states of relative adrenocortical insufficiency.

With this basic frame of reference in mind, a study of the relationship between the degree of stress and the amount of hormone required to meet the stress was undertaken in this series of experiments.

The problem was approached by first establishing the physiological maintenance dose of cortisone for adrenalectomized mice under relatively non-stressful conditions. This maintenance dose was found to be between 0.01 mg. and 0.05 mg. of cortisone acetate/20 gm. of body weight (Kumagai, 1950; Kumagai and Dougherty, 1951). Stress hormone relationships were then studied by pretreating adrenalectomized mice with graded amounts of cortisone acetate including the physiological maintenance dose prior to the administration of standardized stress stimuli.

The data indicate that there is a quantitative interrelationship between hormone level and degree of lymphocytotic response following stress. It is evident from Figure 1 that the degree of the lymphocytotic response induced by the first and second histamine injections is inversely proportional to the amount of hormone administered previously. A possible explanation for the fact that there was no longer a significant difference in the lymphocytotic responses of the 0.005 mg. and 0.05 mg. groups after the third and fourth stresses is that hormone was utilized following the previous histamine injections. Thus the animals of the two groups were in similar insufficient states. Such an explanation would imply that adrenocortical hormone is available to adrenalectomized animals for days following discontinuance of hormone administration. This point will be discussed subsequently.

The quantitative relationship between the amount of hormone and degree of stress is also demonstrated by the percentage of deaths following successive histamine injections (Fig. 2). The number of deaths was inversely proportional to the amount of hormone administered during the pretreatment period.

The fact that previous administration of adrenocortical hormone continues to protect against stress and that the degree of lymphocytotic response is inversely proportional to the amount of hormone administered indicate that hormone is available to an adrenalectomized animal even after administration has been discontinued. The data suggest that the remaining hormone can be mobilized from or activated by stress effects on extra-adrenocortical site(s) to act upon lymphocytes and to protect against lethal effects of large doses of histamine. This is evidenced by two observations following pretreatment with excessive amounts of cortisone. First, a typical lymphocytotic response following stress (indication of relative adrenal cortical insufficiency) was not manifest in the 0.5 mg. group until after the fourth injection of histamine. Animals which had been pretreated with physiological maintenance doses or less of hormone showed typical lymphocytotic responses following the first administration of the stress stimulus. A second observation was that an appreciable percentage of animals in the 0.5 mg. group did not die until after the fourth histamine injection (8 days following discontinuance of cortisone therapy), whereas 30% of the 0.005 mg. group died after the initial injection. Preliminary investigations now in progress indicate that fibroblasts may act as one of the major sites of hormone localization.

The curve of the lymphocytotic response suggests the possibility that a steady state relationship exists between the vascular concentration and tissue concentration of adrenocortical hormone. This relationship can be explained by first defining the five phases of the curve demonstrated by the response of circulating lymphocytes to stress (Fig. 3). During Phase I there is an increase in number of lymphocytes immediately following stress in both intact and adrenalectomized animals (Michael, 1949; Dougherty and Frank, 1953). Phase II represents the lymphopenia which is mediated by pituitary adrenocrotical secretions in intact animals. This phase is absent in adrenalectomized animals. Phase III represents the time of reconstitution of circulating lymphocytes from the maximal lymphopenic level to normal limits. During Phase IV there is a lymphocytotic overshoot that is mediated by non-adrenocortical substances released or activated by stress. Phase V represents the reconstitution of circulating lymphocytes from the maximal lymphocytotic level to their respective pre-stress levels.

It is possible to surmise that "exposure to a variety of stresses increases

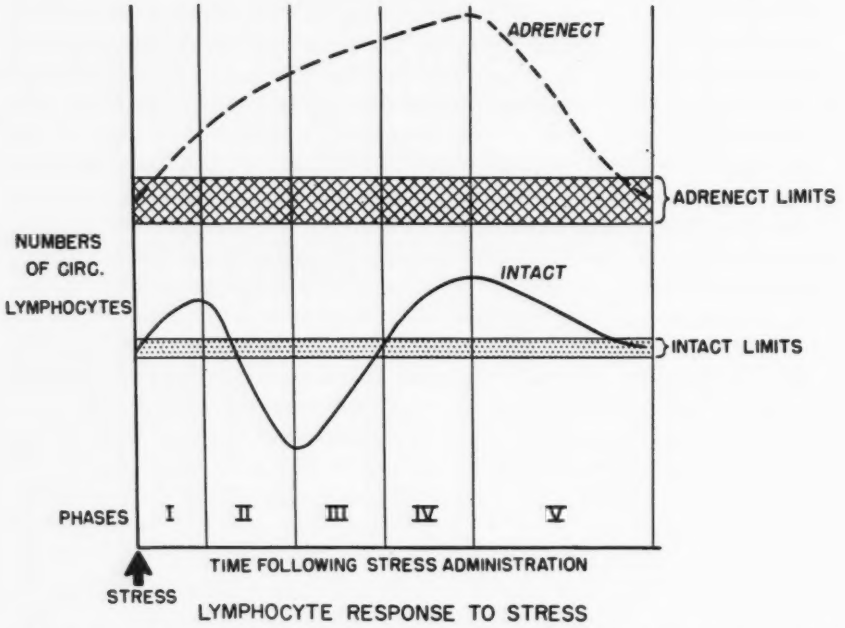

FIG. 3. Comparison of the various phases of the curves of lymphocyte responses of intact and adrenalectomized mice to stress. The phases are discussed in the text.

the tissue utilization of cortical hormones" (Long, 1947), thereby resulting in a brief period of disequilibrium (Lag Phase) between vascular concentration and tissue concentration of hormone. It is proposed that the lag phase occurs because the rate of hormone transfer from the vascular compartment to the tissue compartment proceeds more rapidly than in the opposite direction during this period. The lag phase is represented by Phase I on the lymphocyte curve in Figure 3. The decreased blood level of adrenocortical hormone causes pituitary adrenocortical stimulation according to Sayers (1950) and leads to Phase II. Following stress, it was suggested that there is a release of or action of as yet unknown lymphocyte growth promoting factor (non-adrenocortical factors) resulting in Phases III and IV. During Phase V, the non-adrenocortical factors are balanced after hormone equilibrium is established and consequently the numbers of circulating lymphocytes return to their pre-stress levels.

It is possible that the lymphocytotic response or the lymphocyte reaction to stress stimuli may be developed clinically as a stress tolerance test to determine the capacity of the individual to react to stress stimuli. Thus, after the administration of a standard stress a normal individual would

demonstrate a lymphopenic response, whereas, an individual who is in a state of relative adrenocortical insufficiency would not respond with as marked a lymphopenic response but may even respond with a lymphocytosis depending on the relative degree of insufficiency.

SUMMARY

The data presented indicate that a stress-induced lymphocytosis, referred to as "Lymphocytotic Response," not only occurs in the absence of the adrenal cortex, but may even take place when a relative adrenocortical insufficiency exists, i.e., when the amount of available hormone is insufficient simultaneously to maintain lymphatic tissue and blood lymphocytes within normal limits and to meet the increased demands induced by stress.

The relationship between the degree of stress and adrenocortical hormone requirements was analyzed quantitatively in adrenalectomized CBA mice by studying blood lymphocyte responses. A standardized stress (histamine diphosphate at a dose of 750 mg./kg.) was administered to adrenalectomized mice that had been pretreated with graded amounts of cortisone acetate daily for 8 days. Successive histamine injections were given at 2-day intervals without further hormone treatment. Hematologic values and percentage mortality following successive stresses indicate that adrenocortical hormone may be "stored" in extra-adrenocortical sites after prolonged administration to adrenalectomized mice.

The various phases of the lymphocytic response in intact and adrenalectomized mice to stress is discussed.

The data indicate that the degree of lymphocytosis following stress is inversely proportional to the amount of adrenocortical hormone available to the organism. The possibility that the stress-induced lymphocytotic response may be used as a clinical evaluation of relative adrenocortical insufficiency is discussed.

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EFFECTS OF CORTISONE ON THYROID FUNCTION IN WHITE LEGHORN COCKERELS[1,2]

C. J. SHELLABARGER

Division of Pathology, Medical Department, Brookhaven National Laboratory, Upton, Long Island, New York

INTRODUCTION

ALTHOUGH cortisone has been shown to depress thyroid collection of radioactive iodine in rats by Money *et al.* (1950), Money *et al.* (1951), Perry (1951), Paschkis *et al.* (1952), Albert *et al.* (1952), Rawson (1952), Vezar and Vidovic (1952), Ingbar (1953), Ingbar and Chandler (1953) and Epstein *et al.* (1953), in rabbits by Myant (1953) and in man by Hill *et al.* (1950), Wolfson *et al.* (1950), Frederickson (1951), Frederickson *et al.* (1952), Kuhl and Ziff (1952), Berson and Yalow (1952) and Zingg and Perry (1953), the data on the mechanism whereby this reduced uptake is accomplished are not conclusive. The present report describes experiments designed to determine whether a similar effect occurs in the chick thyroid and to provide data which might suggest the possible mechanism involved.

METHODS

White Leghorn males were given 3 hormone injections spaced 12 hours apart beginning the evening they were hatched. Twelve hours after the third injection they were given 1 microcurie of carrier-free I^{131} in a 0.2 ml. volume and 5 hours later they were decapitated while anesthetized. The thyroids were dissected free, weighed on a microtorsion balance and placed in vials containing 2 ml. of Bouin's fluid. The thyroids were assayed for radioactivity in a well-type scintillation counter to determine percentage of administered dose in the thyroid. The thyroids were then imbedded, cut at 5 microns, stained, and cell heights were determined by the method of Rawson and Starr (1938).

The cortisone employed was Compound E alcohol,[3] administered as a suspension in gum arabic, 10 mg. total dose. TSH[4] was administered in the same carrier in the amount of 1 Junkman Schüller Unit (JSU) total dose, while the controls received gum arabic. All injections were by the subcutaneous route in the back of the neck, except the TSH

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[2] Presented in part before the American Society of Zoologists, Boston, December, 1953.

[3] Cortisone alcohol generously contributed by Doctor H. J. Robinson, Merck Institute for Therapeutic Research, Rahway, New Jersey.

[4] TSH generously contributed by Wayne Donaldson, Parke-Davis & Company, Detroit, Michigan.

TABLE 1. RESULTS OF 10 MG. OF E ALCOHOL IN THE PRESENCE OR ABSENCE OF 1 JSU OF TSH ON THYROID WEIGHT (MG.), CELL HEIGHT (MICRONS) AND I^{131} UPTAKE (PER CENT OF ADMINISTERED DOSE)

Treatment	N	Mean weight	Mean C.H.	Mean uptake	Mean uptake per mg.
TSH	11	5.04*	7.1*	22**	3.1**
TSH+E alc.	13	4.20**	6.2**	12**	2.0
E alcohol	13	2.96	2.4	3**	1.2**
Control	13	2.86	2.5	6	2.4

* = 5% and ** = 1% level of confidence when mean tested against mean immediately below it by t-test.

which was injected on the ventral surface of the neck. Hormone injections were in the volume of 0.1 ml. per injection. During the course of the experiment the birds remained in the chick-boxes without food or water.

In a second experiment, the above protocol was changed as follows: The chicks were one day old when the experiment was begun, the E alcohol was given at the level of 15 mg. and E acetate[5] was administered in the total dose of 15 mg.

A third experiment followed the above procedure in regard to schedule of injections for the E acetate and controls, but in addition, another group received 1.2 mg. of thiouracil. The thyroids were fractioned by a modification (after homogenation) of the $Zn(OH)_2$ procedure of Lutwak, Kuttner and Hankes (1954) and tissue protein-bound I^{131} was determined.

RESULTS

TSH caused an increase in thyroid weight, cell height and I^{131} uptake. E alcohol slightly reduced the thyroid weight and cell height responses to TSH and significantly reduced the uptake of I^{131} per gram of thyroid gland in the presence or absence of TSH (Table 1). E acetate also reduced I^{131} uptake (Table 2). However, E acetate did not prevent the organic binding of I^{131} while thiouracil did (Table 3).

TABLE 2. RESULTS OF 15 MG. OF E ACETATE OR E ALCOHOL ON PERCENTAGE OF ADMINISTERED I^{131} COLLECTED PER MG. OF THYROID

Treatment	N	Mean % uptake per mg.
E acetate	14	1.8**
Control	14	2.5**
E alcohol	12	1.3

** = 1% level of confidence when mean tested against mean immediately below it by t-test.

DISCUSSION

The slight difference between the TSH and TSH plus cortisone treated birds in regard to thyroid weight and cell height are probably not significant. Halmi (1952) studying the effects of TSH administration in the presence or absence of E acetate to hypophysectomized rats, noted no difference in thyroid weight or cell height. Catz *et al.* (1953) noted an increase

[5] Cortone acetate, Merck Institute for Therapeutic Research.

TABLE 3. RESULTS OF 15 MG. OF E ACETATE OR 1.2 MG. OF THIOURACIL UPON PERCENTAGE OF TOTAL THYROID I^{131} IN $Zn(OH)_2$ PRECIPITATE

Treatment	N	Mean % of total I^{131} in $Zn(OH)_2$ ppt.
E acetate	12	82
Control	12	75
Thiouracil	12	45

in cell height of intact E acetate treated rats, as did Higgins *et al.* (1951). No explanation is at hand for the difference in results between these observations and the ones here reported other than that in the present case a relatively large dose of the more soluble E alcohol (Porter and Silber, 1953) was administered.

The finding of a reduced I^{131} uptake in cortisone treated birds is in agreement with similar observations in mammals. The mechanism of this action of cortisone remains in doubt. It is unlikely that cortisone acts to suppress the secretion of TSH which then causes a reduced uptake of I^{131} for the following reasons: The birds of the age used in this study are known to secrete only minute quantities of TSH (Shellabarger and Godwin, 1954). Ingbar and Chandler (1953), Epstein *et al.* (1953), Paschkis *et al.* (1952), Woodbury *et al.* (1951) and Vezar and Vidovic (1952) have shown that cortisone inhibits the expected increase of I^{131} uptake of TSH treated hypophysectomized rats. In addition, Halmi and Barker (1952) have suggested that increased amounts of circulating TSH are found in cortisone treated rats, as based on observations of thyroid histology. D'Angelo *et al.* (1953) reported higher serum TSH titers in cortisone plus thiouracil. treated rats than in rats treated with thiouracil alone. Both Albert *et al.* (1952) and Perry (1951) found no change in thyroid secretion rate in cortisone treated animals. These results indicate that cortisone can act *after* the secretion of TSH to lower thyroid collection of I^{131}.

The failure of cortisone to inhibit the ability of the thyroid gland to trap iodide (Halmi *et al.*, 1953) or organically bind I^{131} as shown in the present study, or synthesize thyroxine as shown by Perry, make the hypothesis that cortisone does act directly on the thyroid gland less secure. Further, there are conflicting reports concerning the ability of cortisone to increase the renal clearance of I^{131}, thereby lowering the amount of I^{131} available for thyroid uptake (Woodbury *et al.*, 1951; Berson and Yalow, 1952; and Ingbar and Chandler, 1953). However, it seems clear from the report of Zingg and Perry (1953) that cortisone did not change renal clearance of I^{131} in man. If this be true, one can then interpret the action of cortisone to be a direct action on the thyroid gland. This interpretation is also possible in the present study, since cortisone depressed the uptake of I^{131} in the pres-

ence or absence of TSH. However, the present study does not exclude the possibility that cortisone might affect the renal excretion of I^{131}, iodide space, utilization of thyroid hormone as suggested by Bondy and Hagewood (1952), circulation time or extra thyroidal metabolism of TSH.

SUMMARY AND CONCLUSIONS

1. In the starved, non-watered, 2- or 3-day old White Leghorn cockerel, cortisone (E alcohol) inhibited the thyroidal uptake of radioactive iodine in the presence or absence of TSH.

2. E acetate was also effective in the latter respect.

3. The mechanism of cortisone action was discussed, the data were interpreted as not being the result of pituitary inhibition of TSH but rather as a direct action of cortisone on the thyroid gland, although other mechanisms cannot be excluded.

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SIMULTANEOUS RELATIVE HYPER- AND HYPO-CORTICOIDISM IN DIFFERENT REGIONS OF THE SAME INDIVIDUAL[1]

HANS SELYE

Institut de Médecine et de Chirurgie expérimentales, Université de Montréal, Montreal, Canada

EARLIER investigations gradually led us to the concept that an important factor in the pathogenesis of many diseases is the failure to adjust the blood-corticoid level to the special requirements created by the evocative pathogen. Maladies, in which such an inadequate adjustment of corticoid secretion is the decisive factor, were thought to represent an important group among the "diseases of adaptation" (Selye, 1950, 1951, 1952; Selye and Horava, 1952, 1953).

However, it is difficult to reconcile with this hypothesis that, by all available assay methods, the corticoid concentration in blood and urine may be virtually normal in a variety of inflammatory conditions, which we considered to be diseases of adaptation.

It is also apparently incompatible with such a humoral concept of pathogenesis that lesions, which had been ascribed respectively to hypo- or hypercorticoidism may be present simultaneously in the same individual, for instance, in a patient with concurrent rheumatoid arthritis and active pulmonary tuberculosis. Here, the inflammation around the joints is excessive and antiphlogistic therapeutic measures are advantageous, while in the lung, the development of the inflammatory granulomatous barricade is manifestly insufficient to protect the patient against the spread of the microbes.

The importance of this last objection was re-emphasized by the demonstration that gluco-corticoids (which are antiphlogistic), such as cortisone, exert a dual effect upon the reaction to topical irritants, in that they inhibit inflammation, but facilitate necrosis (Selye, 1953a). Several investigators consider the initial necrosis, caused by a topical irritant, to be an integral part of the inflammatory process (Ehrich, 1953; Nettleship, 1953; Menkin, 1953). It is difficult to see how a lesion primarily characterized by tis-

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sue-necrosis could be ascribed to a relative local excess, or one with predominantly granulomatous changes to a relative local deficiency of antiphlogistic hormones, if both necrosis and granuloma formation are merely two manifestations of the same condition, namely inflammation.

The object of this communication is to report upon a series of recent experiments designed to clarify this problem in two ways. Firstly, they were to verify, under varied conditions, whether the necrosis-producing effect of a given local stressor can be enhanced, while its ability to cause inflammation is simultaneously depressed, by an antiphlogistic corticoid. Secondly, they were to furnish concrete experimental proof that, under suitable conditions, a relative hyper- and hypocorticoidism can co-exist in different regions of the same animal organism.

MATERIALS AND TECHNIQUES

As *experimental animals*, we used 50 female Sprague-Dawley rats, weighing 145–170 gm. (average 162 gm.). These were subdivided into five groups of ten animals each, as outlined in Table 1.

The *granuloma-pouch* was formed in the usual manner (Selye, 1953b), but with such modifications as were necessitated by the fact that, for comparative purposes, two pouches had to be produced in each animal. These structures, designated respectively as the "cranial" and the "caudal" pouch, are illustrated in Fig. 1.

The cranial pouch was prepared (on the fourth day of the experiment), under light ether anesthesia, by insufflating, through a No. 27 injection needle, 15 ml. of air subcutaneously, in the region between the neck and the shoulder blades. This was followed by the immediate injection, through the same needle (without withdrawing it) of 0.5 ml. of an 0.25% croton-oil solution in corn oil.

The caudal pouch was prepared (on the tenth day of the experiment) in essentially the same manner, by the injection of 15 ml. of air, under the skin of the back in the lower thoracic region, this being followed by 0.5 ml. of a 2% solution of croton oil in corn oil, again without withdrawing the needle. While the rat was still under anesthesia, the same amount of the 2% croton-oil solution was now also injected into the cranial pouch which, by this time, was already lined by a noticeable granulomatous barricade.

As an *antiphlogistic hormone*, we used cortisol,[2] in the form of "hydrocortone" acetate microcrystals, as distributed by Merck & Co. Limited. The hormone was always injected under the ventral skin, at a distance from the pouches, the individual dose being suspended in 0.2 ml. of Merck's suspending fluid. Group I received no hormone, Group II 0.5 mg., Group III 1.0 mg., Group IV 2.5 mg., Group V 5.0 mg., per day, beginning on the first day of the experiment (four days before the granuloma-pouch was prepared) and continuing until the animals were sacrificed, on the 13th day.

The *degree of inflammation* was appraised by the amount of hemorrhagic exudate formed. This was approximately estimated by inspection (while transilluminating the pouch with an electric flashlight) at various times during the experiment; it was then

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[2] In agreement with the suggestion made by Shoppee (1953), the term cortisol is used here in preference to "hydrocortisone" since it eliminates confusion with 4,5-dihydrocortisone and obviates the possible implication that "hydrocortisone" is to be regarded primarily as a mere derivative of cortisone.

accurately measured by aspiration into a graduated syringe, after the animals were killed.

The *degree of necrosis* was determined by the planimetric method described elsewhere (Selye, 1953a).

RESULTS

The results of these experiments are summarized in Table 1 and illustrated in Figure 1.

TABLE 1. EFFECT OF SYSTEMIC CORTISOL TREATMENT UPON AN ACUTE AND A CHRONIC INFLAMMATORY FOCUS, PRODUCED IN THE SAME ANIMAL BY TOPICAL APPLICATION OF CROTON OIL

Group	Daily dose of cortisol in mg.	Pretreated (cranial) pouch		Not-pretreated (caudal) pouch	
		Exudation in ml.	Necrosis extent in % (Incidence in %)	Exudation in ml.	Necrosis extent in % (Incidence in %)
I	None	16.8±3.3	0 (0)	3.2±0.42	2.2± 0.67 (10)
II	0.5	15.0±0.6	0 (0)	1.8±0.41	20.9± 5.7 (80)
III	1.0	6.5±1.5	0 (0)	0.5±0.27	33.0± 6.4 (90)
IV	2.5	2.4±0.65	0 (0)	0.2±0.16	60.0± 7.86 (100)
V	5.0	3.4±1.6	0 (0)	0.2±0.22	53.4±10.4 (100)

The data in Table 1 refer exclusively to observations made on the 13th day, after the animals were sacrificed. The mean volume of the exudate and the mean area of necrosis are listed (together with the standard errors), as well as (in brackets) the incidence of necrotic pouches in any one group, irrespective of the area involved.

It should be mentioned that, although none of the cranial pouches exhibited necrosis after the injection of the 2% croton-oil solution, several (one rat in Group II, two rats in Group III and three rats each in Groups IV and V) could not be reinjected because they became necrotic after the first injection of the initial dose of 0.25% croton oil, evidently because the cortisol treatment predisposed the tissues to necrosis.

Table 1 shows that, in the controls which received no hormone injection, the croton oil pretreated (cranial) pouches responded with considerable inflammatory exudate formation (16.8 ml.). Repeated transillumination during the experiment revealed that part of this fluid had already been formed as the result of the initial dilute croton-oil solution, although much additional exudation occurred after the concentrated solution was injected. In the hormone treated groups, exudate formation is increasingly more suppressed, as the dose of the hormone rises, up to the maximally effective dose of 2.5 mg. per day. On the other hand, necrosis did not occur after the injection of the strong croton-oil solution in any of these pretreated pouches.

In the not croton-oil pretreated (caudal) pouches, exudation was less pronounced since here, the irritation, though more intense, was of lesser

duration. Nevertheless, the inhibition of exudate formation is again increasingly more pronounced, within the range of 0 to 2.5 mg. per day, as the dose of hormone rises. Conversely, the extent and the incidence of necrosis are negligible in the not hormone-treated controls (one animal out of ten had a small area of necrosis), but they are increasingly more pronounced as the daily dose of cortisol is raised.

In other words, the more intense the cortisol treatment, the more mark-

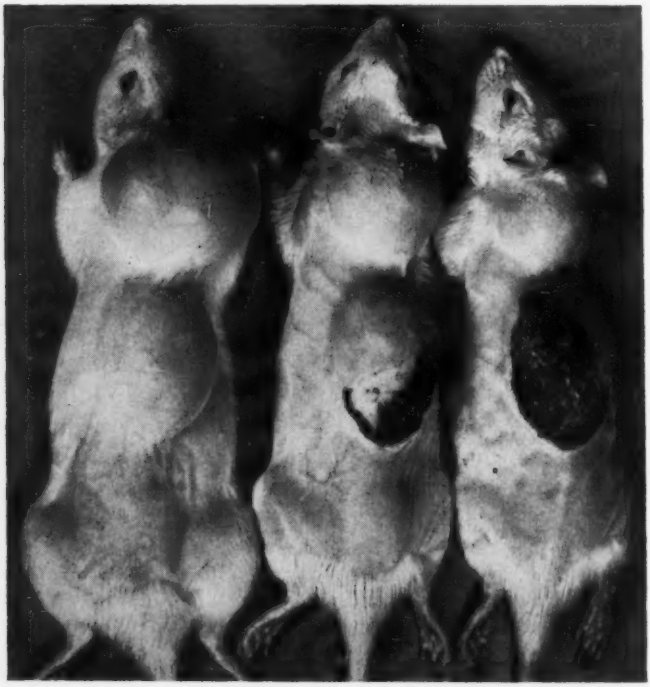

FIG. 1. *Effect of systemic cortisol treatment upon an acute and a chronic inflammatory focus, produced in the same animal by topical application of croton oil.*—Reading from left to right. *Rat receiving no hormone treatment* (Group I). Both pouches show great distention by exudate, but no sign of necrosis. *Rat receiving 1 mg. of cortisol per day* (Group III). The pretreated (cranial) pouch shows less inflammatory exudation, but part of the not-pretreated (caudal) pouch has become necrotic. This is the condition interpreted as co-existent "relative regional hypo- and hypercorticoidism" since, in the cranial pouch, disease-manifestations (exudation and inflammation) were not sufficiently suppressed, while in the caudal pouch, they were actually aggravated by the blood-cortisol level induced in this animal. *Rat receiving 5 mg. of cortisol per day* (Group V). Virtually complete inhibition of the local lesions in the cranial pouch ("relative regional eucorticoidism"), while the caudal pouch tissues underwent complete necrosis, as a direct result of a blood-corticoid level, which was far too high for the intense, acute topical stress induced in this region.

edly exudation is inhibited and necrosis enhanced. If we consider the efficacy of such treatment in suppressing the "disease-manifestations," it may be said that the best results were obtained in the cranial pouches of Groups IV and V. Here inflammation was almost completely inhibited and yet necrosis failed to occur. All other foci were less favorably influenced, either because there was more inflammation or more necrosis.

DISCUSSION

Exudation is inhibited if appropriate amounts of antiphlogistic hormones are secreted. We can therefore view exudation as a manifestation of a local hypocorticoidism, in the sense of a lack of antiphlogistic corticoids, in relation to the amount that would be required to maintain regional homeostasis despite topical stress. For similar reasons, in these experiments, necrosis can be viewed as an indication of a relative, local hypercorticoidism. It may be concluded that, in this same sense, both the cranial and the caudal pouches suffered from an induced regional, relative hypocorticoidism, in the rats receiving no hormone treatment. Here, the tissues could have been maintained in a more normal condition by a higher blood-level of antiphlogistic corticoids.

The rats receiving the highest doses of cortisol showed an essentially eucorticoid condition in their cranial pouches. Their caudal pouches, however, exhibited intense hypercorticoidism in relation to requirements for optimal homeostasis in the face of the existing intense topical stress situation. They underwent actual necrosis, under the combined influence of the strong croton-oil solution and of the systemic cortisol treatment.

In this same sense, the animals receiving the smaller doses of cortisol showed relative regional hypocorticoidism in their cranial, and hypercorticoidism in their caudal pouches. Yet, the blood supplying these two regions must have contained the same concentration of cortisol, since the hormone was administered systemically.

It will be remembered that, according to our stress-concept (Selye, 1953c), the diseases of adaptation are caused by an essentially tripartite pathogenic situation, rather than by any one agent. (1) The "eliciting pathogen" (microbes, drugs, physical agents), (2) the prophlogistic and (3) the antiphlogistic factors are equally important in principle, although one or the other may be the decisive factor in any one case. In the present experimental series, the eliciting pathogen corresponded to the croton oil, the antiphlogistic hormone to cortisol and the blood-level of prophlogistic factors was not altered. Nevertheless, essentially different disease-manifestations were induced, by merely varying the intensities and the time-relations of the treatment with the first mentioned two factors.

Of course, even this hypothesis of the "tripartite pathogenesis" of dis-

ease recognizes the existence of "unconditional pathogens," that is of agents so toxic to the organism that their immediate effects cannot be prevented by adaptive measures. For example, severe X-irradiation and intense mechanical or chemical trauma belong to this class. Naturally, there are many intermediate types between the entirely unconditional pathogen and that highly subject to the influence of adaptive reactions. The stronger of our two croton-oil solutions comes close to being an unconditional pathogen. Normally it causes intense inflammation and any effort to inhibit this disease-manifestation, by antiphlogistic hormone therapy, immediately leads to necrosis. Therefore, in the unpretreated (caudal) pouches, a complete inhibition of the lesions was never accomplished and, by giving cortisol, we merely substituted necrosis for exudation.

On the other hand, by pretreating the connective tissue with a very dilute solution of croton oil, the phenomenon of local resistance (Selye, 1953d, 1953e) was induced in the cranial pouch, so that the strong croton-oil solution, subsequently applied, was less toxic to this region than to the unpretreated area of the caudal pouch. Consequently, in the pretreated (cranial) pouch, it became possible to inhibit exudation with cortisol without incurring the danger of necrosis. Here, we have a clear-cut example illustrating that, in the same organism, there may be one region in which the availability of corticoids is the decisive factor in the genesis of pathologic manifestations, while in another area, the effects of the same pathogen cannot be suppressed by any adjustment of the blood-corticoid level.

It would serve no useful purpose to discuss the possible clinical implications of these findings at length. Evidently, one is led to ask whether differences in the regional "hormone-utilization rate" or in the hormone-affinity of tissues could account for regional differences in the course of various inflammatory and necrotizing lesions. Such topical changes in hormone-metabolism could result in a simultaneous, *absolute* regional hypo- and hypercorticoidism within the same patient. There are no conclusive data to prove that these conditions actually exist. In our opinion, the experiments just reported definitely prove, however, that the duration and intensity of topical stress can so alter the local hormone-requirements, for optimal tissue-homeostasis, that a *relative* hypo- and hypercorticoidism can co-exist in adjacent connective-tissue territories of the same animal.

SUMMARY

A modification of the granuloma-pouch technique is described. This preserves the advantages of the original procedure (possibility of accurate delimitation of a regular connective-tissue area, objective assessment of local stress by the volumetric measurement of exudation and planimetric determination of necrosis) and permits comparative studies in two regions of the same individual.

Using this "double granuloma-pouch" technique, one test area of connective-tissue was rendered relatively resistant by pretreatment with small doses of croton oil, and then a high concentration of this same irritant was introduced into both areas.

Not hormone-treated control rats responded only with strong inflammation in both areas. The response was markedly altered, however, in rats which received daily treatment with hydrocortisone, at various dose levels. In the pretreated pouches, the inflammatory phenomena were increasingly more inhibited as the dose of hydrocortisone rose and yet necrosis did not occur. Thus it proved possible to adjust the blood-corticoid level satisfactorily since, with the highest doses, topical disease-manifestations were almost completely suppressed.

In the pouches which had not been protected by pretreatment with the low concentration of croton oil, the subsequently applied stronger solution produced inflammation and exudation in the controls while, in the rats receiving cortisol, necrosis ensued. The latter was roughly proportionate in extent to the amount of hormone given.

Thus it became possible to induce a relative hyper- and hypocorticoidism simultaneously in different regions of the same animal, in that a given dose of hydrocortisone proved insufficient to suppress the lesions completely in one focus, although it actually aggravated them in an adjacent area.

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NOTES AND COMMENTS

INFERTILITY IN RATS AND MICE FOLLOWING ESTROGEN TREATMENT EARLY IN LIFE[1]

Several workers have reported that the administration of estrogen to female rats before the 15th day of life caused permanent changes in the genital tract (Green and Burrill, 1941; Turner, 1941; Wilson, 1943; Hale, 1944). Ovarian function was permanently deranged and consequently the animals were infertile. The amount of estrogen required to produce these effects was quite small, but information on the minimal effective dose was not available. In mice, Leathem and Merklin (1950) and Merklin (1953) found that a single injection of 10 μg. estradiol dipropionate at 10 days of age induced sterility in about half the animals. The cause of the infertility was not apparent, however, for ovarian function appeared normal. In the present study, the lowest effective dose for rats was ascertained, and evidence was adduced on the mechanism of the infertility in mice.

Female albino rats were given a total of 4, 20, or 100 μg. estradiol dipropionate in peanut oil. The doses were given in 12 injections spread over 4 weeks from the 4th day of life. A control group of rats was given similar injections using oil only. When the rats were 10 weeks old they were placed with males for three months, after which they were sacrificed. The number of animals that bore litters is shown in Table 1.

TABLE 1. THE FERTILITY OF FEMALE RATS AND MICE INJECTED PREPUBERALLY WITH ESTRADIOL

Species	Total dose of estrogen in μg.	Number of animals	
		Mated	Fertile
Rat	—	7	7
	4*	8	7
	20	6	0
	100	9	0
Mouse	—	14	14
	5†	13	1‡

* Estradiol dipropionate.
† Estradiol benzoate.
‡ Pregnant when killed.

The results indicate that the minimal effective dose for the rat lies between 4 and 20 μg. estradiol dipropionate. Thirteen of the fifteen infertile rats were found at autopsy to have large, purulent, tubal cysts. In some animals one or both ovaries had apparently been engulfed in the cysts. Ovaries from 10 infertile animals were examined histologically; only one contained corpora lutea. Cystic endometrial hyperplasia was present in three rats. These pathological findings are similar to those reported by Hale (1944). However,

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[1] This material formed part of a M.Sc. thesis presented at the University of Western Australia.

in the present experiments estrus was noted only irregularly in the treated rats, whereas many of his rats evinced continuous estrus.

Albino female mice were injected from the second day of life for 4 weeks. They received a total dose of 5 μg. estradiol benzoate in peanut oil in 12 injections. Another group of mice was given a similar series of injections using oil only. When they were 9 weeks old, all the mice were placed with males for a period of 4 months, after which they were killed.

It was found that 12 of the 13 treated mice were permanently sterile (Table 1). The greater degree of sterility induced by this treatment as compared with that of Leathem and Merklin (1950) and Merklin (1953) was probably related to the earlier commencement of treatment in the present study. Cystic endometrial hyperplasia was present in 10 of the infertile mice, and metaplasia of the uterine epithelium was also seen in 3 animals. In 5 of the 13 mice the histological appearance of the ovaries indicated they had been functioning normally; in the remainder no corpora lutea were seen, though there were numerous follicles of medium size.

It is concluded that the primary cause of infertility in most of the animals was a disturbance of pituitary function, but in those animals in which the ovaries appeared normal the infertility was probably due to abnormalities found in the genital tract—cystic endometrial hyperplasia in the mice, and cystic fallopian tubes in the rats. A similar persistent infertility associated with cystic endometrial hyperplasia and normal ovarian function has been reported in sheep that had been injected with stilbestrol (Underwood, Shier and Peterson, 1953) or fed on estrogenically-potent subterranean clover (Bennetts, Underwood and Shier, 1946; Underwood and Shier, 1951).

SUMMARY

Persistent infertility was induced in female rats by a total dose of 20 or 100 μg. estradiol dipropionate spread over 4 weeks from the 4th day of life, but a dose of 4 μg. was ineffective. In mice, a total dose of 5 μg. over 4 weeks from the 2nd day of life induced sterility. The infertility was referable to failure of ovulation in most animals, but in some mice the infertility could only be attributed to the cystic glandular hyperplasia that was regularly seen in the endometria of infertile mice.

A. W. H. Braden[2]

W. A. Department of Agriculture
Animal Health and Nutrition Laboratories
Nedlands
Western Australia

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[2] Present address: Division of Animal Health and Production, C.S.I.R.O.; McMaster Laboratory, Sydney, N.S.W., Australia.

THE EFFECT OF PREGNANCY ON THE EXCRETION OF WATER LOADS BY RATS[1]

The effect of pregnancy on water metabolism remains a still but poorly delineated phenomenon; a like situation obtains in the evaluation of the role of alterations in water content and distribution in the etiology of the toxemias of pregnancy (Pickford, 1952). Intimately connected with this general problem is the nature of the endocrine activity of the uterine contents, for there are many reports which attest to a wide variety of hormonal influences on water metabolism (Gaunt and Birnie, 1951).

The rate of excretion of a test load of water has been used as an index of adrenal cortical function (Gaunt *et al.*, 1949) and as a means for the detection of antidiuretic substances (Burn, 1931 and Ames *et al.*, 1950, among others). The water-loading situation has accordingly been employed in this study to determine if pregnancy affects the rate at which rats dispose of excess water. For the better elucidation of possible placental and/or fetal influences, observations were made on hypophysectomized as well as intact pregnant and non-pregnant animals.

MATERIALS AND METHODS

Female rats of the Sprague-Dawley strain, weighing from 180 to 220 grams were used. They were maintained in an air-conditioned room (temperature: 78 to 80° F.) and had free access to ground Purina Lab Chow and tap water at all times (except for an eighteen hour fast before the time of water-loading). Hypophysectomy was performed on the 12th day of pregnancy, the "intact" rats being subjected to sham operations at this time also. The surgical procedures and methods for verification of completeness of operation as well as the methods employed for the obtaining and the timing of the pregnancies have been described previously (Knobil and Caton, 1953).

The pregnant animals received the water loads (5 ml. tap H_2O/100 gms. body weight, p.o.) on the 19th day of pregnancy. The non-pregnant animals were tested on the sixth post-operative day. The exact procedures employed in these tests have been described previously (Hofmann, 1952). Minute urine volumes were computed on the basis of the urine flows which obtained during the period of time required for the excretion of 40% of the administered water. (This endpoint, rather than the conventional 50% of the dose, was used because many of the hypophysectomized rats had failed to excrete half of their water load by the end of the seven hour observation period).

RESULTS

The urine flows which obtained after the administration of the water loads are reported in Table 1. While a difference between the hypophysectomized and intact rats can be demonstrated ($P < .02$), in neither the intact nor the hypophysectomized rats does pregnancy affect the rate at which a water load is excreted ($P > .1$).

TABLE 1. THE RATE OF EXCRETION OF WATER LOADS BY INTACT AND HYPOPHYSECTOMIZED PREGNANT AND NON-PREGNANT RATS

Physiological status	No. animals	Urine flow[1] (ml./min./100 gm.)	Stand. error of the mean
Intact	8	0.021	±0.003
Intact, Pregnant	10	0.026	±0.006
Hypophysectomized	13	0.008	±0.004
Hypophysectomized, Pregnant	9	0.005	±0.001

[1] During the period of time required for the excretion of 40% of the administered water.

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DISCUSSION

By the criteria employed in this study, no marked effect of pregnancy on water metabolism can be demonstrated. It was hoped that the water-loading situation would afford an opportunity for the maximal expression of any diuretic or antidiuretic influences of abnormal magnitude which were operative in the pregnant rat, especially those which reflected endocrine activity on the part of the uterine contents. If such deviations do indeed exist, it is evident more sensitive means will be required for their identification.

The wide variation encountered in the excretory performances of the hypophysectomized rats is not an uncommon experience. Computations made from the data of Joseph *et al.* (1944), obtained from hypophysectomized rats subjected to similar water loads, reveal an average minute urine volume of 0.005 ml./min./100 gms. but a range of from 0.023 to 0.004 ml./min./100 gms. Similar increases in variation are seen following adrenalectomy (Gaunt, 1946).

SUMMARY

The administration of water loads to intact and hypophysectomized pregnant and non-pregnant rats reveal no marked effect of pregnancy on the rate of disposal of excess water.

FREDERICK G. HOFMANN,[2] ERNEST KNOBIL[3] AND WILLIAM L. CATON[4]
Biological Research Laboratory
Harvard School of Dental Medicine
the Department of Obstetrics
Harvard Medical School
and the Boston Lying-In Hospital
Boston 15, Massachusetts

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[2] National Research Council Fellow in the Medical Sciences. Present address: Dept. of Pharmacology, College of Physicians and Surgeons, Columbia University, New York 32, N. Y.

[3] Milton Fellow, Harvard University. Present address: Dept. of Physiology, Harvard Medical School, Boston, Mass.

[4] Present address: Dept. of Obstetrics and Gynecology, School of Medicine, Emory University, Atlanta, Ga.

DELAYED EOSINOPENIC RESPONSE OF THE GUINEA PIG FOLLOWING INJECTION OF EPINEPHRINE

Administration of epinephrine in the normal guinea pig failed to elicit the characteristic eosinopenic response which occurs in other species 3 hours after injection (Schweizer, 1953). The data indicated that the eosinopenia in the guinea pig was being masked possibly by the outpouring of eosinophils which had occurred one hour after injection and it was suggested that the eosinopenia might occur later in this form as it does in some strains of rats (Gordon, personal communication).

The procedures used to test this possibility were the same as described in the earlier paper (Schweizer, 1953) except that the second blood sample was drawn 6 hours after epinephrine injection.

TABLE 1

Experimental procedure	No. of animals	Polynuclear leucocytes			Mononuclear cells			Eosinophils		
		0 hour	6 hour	Dif. ± S.E. (% change)	0 hour	6 hour	Dif. ± S.E. (% change)	0 hour	6 hour	Dif. ± S.E. (% change)
Normal	10	4,140	4,647	+507 ± 375 (+12.2%)	6,507	5,615	−892 ± 507 (−13.7%)	140	134	−6 ± 17 (−4.3%)
Normal + Epinephrine	10	5,093	12,685	+7,592 ± 1,228[1] (+149.0%)	5,550	3,315	−2,235 ± 376[1] (−40.3%)	108	19	−89 ± 19[1] (−82.4%)

[1] Highly significant change $P<.01$.

The data are summarized in the table. Six hours after the injection of epinephrine (40 γ/100 gm. body weight) there was a consistent and significant fall in the number of eosinophils (−82.4%). The fall in mononuclear cells and the rise in polynuclear cells which had occurred 3 hours after injection were also found at the 6th hour (−40.3% and +149.0%, respectively).

MALVINA SCHWEIZER

Department of Biology
Washington Square College of Arts and Science
New York University
New York, N. Y.

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THE EFFECT OF ACTH ON ADRENAL SIZE IN HYPOPHYSECTOMIZED RATS AFTER REMOVAL OF ONE ADRENAL[1]

INTRODUCTION

Unilateral adrenalectomy has been known for some time to produce hypertrophy of the remaining adrenal in a variety of animals, and this compensatory hypertrophy is abolished by hypophysectomy (Tepperman, Engel and Long, 1943). Most authors have interpreted this data to mean that unilateral adrenalectomy lowers the level of circulating blood corticoids, and that this drop in corticoids stimulates the pituitary to release increased amounts of ACTH, resulting in hypertrophy of the remaining gland. Gemzell, Van Dyke, Tobias and Evans (1951) have shown that bilateral adrenalectomy causes an increased pituitary production of ACTH as well as an increased ACTH level in the peripheral blood, and presumably, unilateral adrenalectomy produces a similar rise. A factor that has been largely ignored in the literature, however, is the fact that the initial mass of adrenal tissue in unilaterally adrenalectomized animals is one-half that of the normal control. It could be argued thus, that at a constant ACTH output, the animal with less adrenal tissue would respond with more hypertrophy because there was less adrenal tissue on which the hormone could act. The following experiments were therefore initiated to determine the degree of hypertrophy occurring when the amount of adrenal tissue is varied in hypophysectomized rats maintained on a constant dose of ACTH.

MATERIALS AND METHODS

One-half of a group of hypophysectomized male Sprague-Dawley rats were subjected to left adrenalectomy one week after hypophysectomy, and the remainder were sham operated.[2] The animals were then given 4 units of ACTHAR GEL (Armour) daily subcutaneously for 6 days, killed on the seventh day, and their adrenals weighed. The same experiment was repeated, injecting with 0, 8, and 16 units of ACTHAR GEL per day. The animals were fed a diet of Purina laboratory chow supplemented with meat and oranges. They were weighed before and after the experiment. Adrenals were weighed to the nearest milligram on an analytic balance and fixed in 10% formalin for histologic examination. At autopsy the sella was examined under a dissecting microscope and the adrenal area examined grossly for adrenal remnants. In all cases hypophysectomy and unilateral adrenalectomy had been complete.

In the sham operated group, eight rats received 0 units per day, eleven 4 U., fifteen 8 U., and sixteen 16 U. In the unilaterally adrenalectomized groups the numbers were seven, nine, fourteen, and sixteen respectively.

RESULTS

If variations in the amount of adrenal tissue present alter the degree of hypertrophy per unit of adrenal tissue in response to ACTH, one would expect more hypertrophy in the rats with one adrenal than in those with both at each ACTH dose level. That this was not the case is shown in Figure 1. It is apparent that the right adrenal weight is essentially the same whether the left adrenal is present or not. Thus, while the total amount of adrenal hypertrophy is greater in animals with two adrenals, the response per unit of adrenal is the same whether one or both adrenals are present. Furthermore, this is true at all dose levels used. Most of the animals lost moderate amounts of weight on ACTH, but at each dose level there was no significant difference between the weight loss in sham operated and unilaterally adrenalectomized animals. The same pattern emerges when the results are analyzed using absolute, rather than relative, adrenal weights. At all dose

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[1] This work was supported by a grant-in-aid from The Commonwealth Fund.

[2] Hypophysectomized rats obtained from Hormone Assay Laboratory, Inc., Chicago, Illinois.

levels, histologic examination of the adrenals confirms the weight data. There is no significant microscopic difference between the right adrenal of the sham operated rats and the unilaterally adrenalectomized animals. (Fig. 2.)

Selye (1947) states in his "Textbook of Endocrinology" that the adrenals, the thyroid and the gonads all respond to their trophic hormones with similar increases in weight per unit of tissue regardless of the amount of tissue initially present, but this dictum has received little attention. The present study amply confirms Selye's statement as far as adrenal responses are concerned.

This observation has a number of interesting implications. In the first place, it implies that "compensatory" adrenal hypertrophy after unilateral adrenalectomy is due entirely to increased ACTH production, since a constant rate of endogenous secretion would produce no change in the size of the remaining adrenal.

Furthermore, this data suggests that the degree of regeneration of an adrenal after incomplete adrenalectomy is controlled by the amount of increased ACTH production, and that it may be wrong to speak, as is often done, of "complete adrenal regeneration," if a few cells are left behind. Ingle and Higgins (1939) found that this was true when they removed varying amounts of adrenal capsule after demedullectomy.

Finally, such data offers indirect evidence that ACTH is not permanently "fixed" or inactivated by adrenal tissue. Assuming that the hypertrophic response of the cortex is proportional to the amount of ACTH available to it, one would expect twice the hypertrophy in the unilaterally adrenalectomized animal if the adrenal were the sole or even the major sight of ACTH removal from the blood stream. Sydnor and Sayer's (1953) observation that the half life of ACTH in the adrenalectomized animal is very short supports this concept also, suggesting that non-adrenal tissues rapidly remove circulating ACTH.

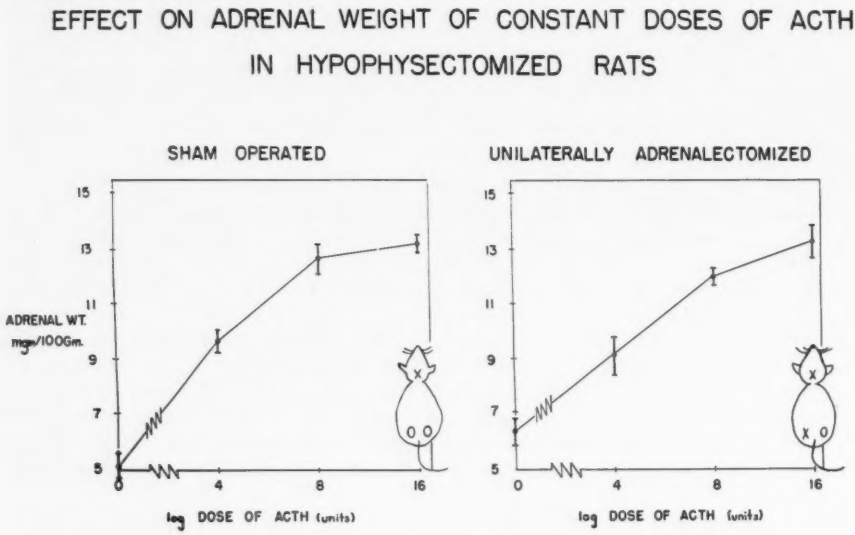

FIG. 1. Right adrenal weight in unilaterally adrenalectomized and sham operated rats. The dots are mean weight values in mg./kg., and the bars represent ± the standard error.

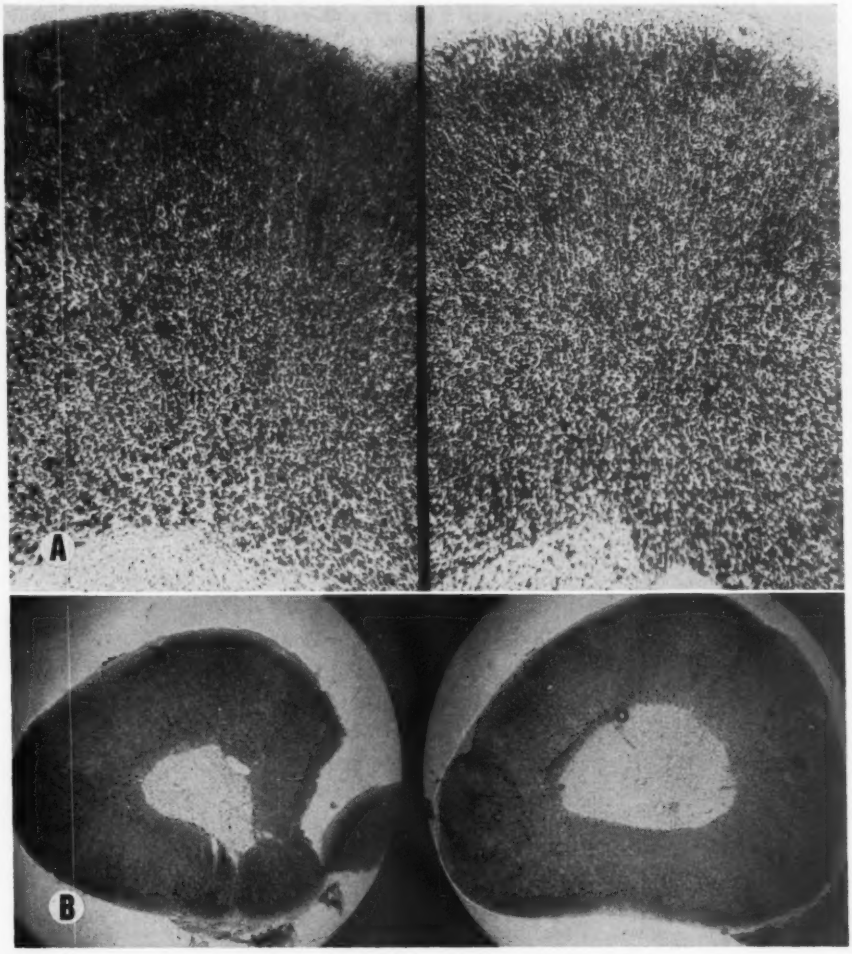

FIG. 2. Right adrenals of hypophysectomized rats receiving 16 μ. ACTHAR GEL per day for 7 days. Left: unilaterally adrenalectomized. Right: sham operated. A: (×135; frozen section, sudan IV-stain) B: (×30; frozen section, sudan IV-stain).

SUMMARY

The adrenal weight response to a constant dose of ACTH in hypophysectomized animals has been determined in unilaterally adrenalectomized rats and sham operated controls.

At 4, 8, and 16 units per day of ACTH, the extent of the hypertrophy of the right adrenal is the same whether the left adrenal is present or not.

It is concluded that the response of adrenal tissue per unit of adrenal is the same

regardless of the total amount of adrenal tissue present, and that, therefore, the amount of tissue present does not condition the response to ACTH.

The implications of this observation are discussed.

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W. F. GANONG[3]

Surgical Laboratory
Harvard Medical School
Boston, Massachusetts

[3] Fellow of the National Institute of Neurologic Diseases and Blindness.

ANNOUNCEMENTS

THE THIRD PAN-AMERICAN CONGRESS IN ENDOCRINOLOGY

Santiago, Chile

November 21–27, 1954

Members of the Endocrine Society are cordially invited to attend the Third Pan-American Congress in Endocrinology. The faculty is composed of eminent investigators in endocrinology from North and South America.

This is an unusual opportunity for you and your family to enjoy a pleasant vacation in South America and for you to attend a highly instructive medical assembly. Correspondence should be directed to Dr. Arturo Atria, Hospital del Salvador, Casilla 70-D, Santiago, Chile. Copy of tentative program may be had from the Secretary-Treasurer, The Endocrine Society, Suite 319, 1200 North Walker Street, Oklahoma City 3, Oklahoma.

GORDON RESEARCH CONFERENCE

July 19, 1954, Matrix formation in bones and teeth, C. Leblond, Chairman: F. L. Losee and W. C. Hess, "Organic components of dentin matrix"; R. E. Clegg, "Carbohydrates of bone and dentin matrix"; D. D. Dziewiatkowski, "Incorporation of radioactive elements into bone and dentin matrix." F. McLean, Chairman; R. M. Amprino, "Bone structural renewal"; J. Gross, "Collagen fibrogenesis."

July 20, Chemistry of mineralization, H. C. Hodge, Chairman: V. DiStefano, "Role of phosphatase"; B. B. Migocovsky, "Isotope studies"; A. E. Sobel, "Local factor in calcification." A. B. Gutam, Chairman: W. F. Neuman, "Theory of calcification"; T. Dixon, "Origin and nature of bone citrate."

July 21, Dynamics of calcium metabolism, W. D. Armstrong, Chairman: D. L. Buchanan and A. Nakao, "Physiological status of bone carbonate"; C. J. Maletskos, "Dynamic model of calcium metabolism"; William P. Norris, "Radiocalcium and radium studies"; M. Rubin, subject to be announced. J. E. Howard, Chairman: H. D. Copp, "Calcium and phosphorus homeostasis."

July 22, Nutritional factors, R. S. Harris, Chairman: J. Cohen, "Newer studies on vitamin A in bone growth"; C. L. Comar, "Calcium metabolism and bone formation"; H. E. Harrison, "Vitamin D in calcium metabolism"; W. van Robertson, "Ascorbic acid in fibrogenesis." Afternoon workshop

—some analytical aspects, J. Sendroy, Jr., Chairman. H. H. Mitchell, Chairman: R. Nicolaysen, "Physiological basis for evaluation of man's calcium requirements." Panel discussion: L. Barnes, H. H. Mitchell, R. Nicolaysen, D. Watkin, "Human requirements for calcium."

July 23, Physiological aspects of bone formation, R. Follis, Chairman: J. Arnold, "Bone remodeling"; G. Bevelander, "Histochemistry of osteogenesis"; P. Munson and A. D. Kenny, "Nature and mechanism of action of parathyroid hormonal activity"; N. S. McDonald, "Some aspects of radio-strontium deposition."

Individuals interested in attending the Conference are requested to send in their applications to the Director. Each applicant must state the institution or company with which he is connected and the type of work in which he is most interested. Attendance at each Conference is limited to 100.

Requests for attendance at the Conferences, or for any additional information should be addressed to W. George Parks, Director, Department of Chemistry, University of Rhode Island, Kingston, Rhode Island. After June 15, 1954, mail should be addressed to Colby Junior College, New London, New Hampshire.

U.S.P. STEROID REFERENCE SUBSTANCES

The need for easily available steroid preparations for use as reference standards in connection with the rapidly developing paper chromatographic techniques was recognized by the Endocrinology Study Section of the Division of Research Grants, National Institutes of Health, as early as 1952.

This situation was brought to the attention of the Board of Trustees of the United States Pharmacopeial Convention. As a result, in April 1953, a U.S.P. Advisory Board on Steroid Reference Substances was established. The membership is as follows: T. F. Gallagher, Sloan-Kettering Institute; Gregory Pincus, Worcester Foundation; Max Tishler, Merck & Co.; Adley B. Nichols, U.S.P.; and Sam R. Hall, Division of Research Grants, N.I.H. (Chairman). This board is responsible for the selection of reference substances, verification of their authenticity, and determination of their suitability.

Karl Pfister of Merck & Co. provided invaluable aid in the coordination of the many technical aspects of this program. The cooperating laboratories are those of Leonard Axelrod of the University of Rochester School of Medicine and Dentistry; Ralph I. Dorfman and S. Burstein of the Worcester Foundation; and Attallah Kappas of the Sloan-Kettering Institute.

Acknowledgment and appreciation for donations of the steroids is expressed to the following: Ciba Pharmaceutical Products, Inc.; The Glidden Company; Lederle Laboratories; Merck & Co. Inc.; Schering Corporation; The Squibb Institute for Medical Research; Syntex, S.A.; and The Upjohn Company.

These compounds are supplied for use only as paper chromatography standards. The contaminants noted (see table) were detected by chromatography of large amounts of the substances, and by employing a variety of chromatographic systems and staining methods. Under ordinary circumstances the presence of these trace contaminants will not hinder use of the standard for the purpose noted.

It was found feasible to dilute the steroids with an inert, inorganic substance from

PHYSICAL CONSTANTS

Set No. 1

Number	*Compounds*	*M. P.*	$[\alpha]_D$	*U. V.*	*Contaminants noted*
5	3α-Hydroxyandrostane-17-one	182–184°	+97.5°(E)	ϵ294mμ=50	One more polar. One less polar.
11	17β-Hydroxyetiocholane-3-one	140–142°			One more polar. Two less polar.
19	Etiocholane-3,11,17-trione	132–135°	+150.8°(An)		Two more polar. One less polar.
23	3α-Hydroxyetiocholane-11,17-dione	186–188°			One less polar.
39	3α,11β-Dihydroxyetiocholane-17-one	233–236°			One more polar.
113	17α-Hydroxy-Δ⁴-pregnene-3,20-dione	212–220°			Three more polar.
161	11β,21-Dihydroxy-Δ⁴-pregnene-3,20-dione	180–184°	+224± 2°(E)	ϵ242mμ=16,300	Two more polar. One less polar.
193	17α,21-Dihydroxy-Δ⁴-pregnene-3,20-dione	206–207°	+125°(C)		Three more polar.
194	3β,17α,21-Trihydroxy-Δ⁵-pregnene-20-one	241–243°			Two more polar. One less polar.
210	3β,21-Dihydroxy-Δ⁵-pregnene-20-one	181–184°			One more polar.
225	11β,17α,21-Trihydroxy-Δ⁴-pregnene-3,20-dione	216–218°		ϵ242mμ=15,900	Two more polar. One less polar.
227	11β,17α,21-Trihydroxypregnane-3,20-dione	213–215°			Two more polar.
228-A	11β,17α-Dihydroxy-21-acetoxyallopregnane-3,20-dione	206–214°	+85.5°(An)		One more polar.
231	3α,11β,17α,21-Tetrahydroxypregnane-20-one	208°	+83°(An)		Two more polar.
241	11α,17α,21-Trihydroxy-Δ⁴-pregnene-3,20-dione	224–225°	+116°(E)	ϵ242mμ=15,500	Two more polar. One less polar.
273	21-Hydroxy-Δ⁴-pregnene-3,11,20-trione	179–183°	+261°(E)	ϵ238mμ=15,800	Two more polar. One less polar.
279	3α,21-Dihydroxypregnane-11,20-dione	221–225°			None detected.
289	17α,21-Dihydroxy-Δ⁴-pregnene-3,11,20-trione	226–228°	+209°(M)	ϵ238mμ=15,800	One more polar.
291	17α,21-Dihydroxypregnane-3,11,20-trione	228–232°	+79.0°(An)		Two more polar.
292	17α,21-Dihydroxyallopregnane-3,11,20-trione	235–238°			Two more polar.
292-A	17α-Hydroxy-21-acetoxyallopregnane-3,11,20-trione	239–241°	+91.7± 1°(C)		One more polar.
294-A	3β,17α-Dihydroxy-21-acetoxyallopregnane-11,20-dione	235–236°			One more polar.
295	3α,17α,21-Trihydroxypregnane-11,20-dione	195–202°	+66.0(D)		None detected.
295-A	3α,17α-Dihydroxy-21-acetoxypregnane-11,20-dione	226–229° (softens at 210°)	+75± 2°(An)		One more polar.

Footnotes:

An = Acetone
C = Chloroform
D = Dioxane
E = Ethyl alcohol
M = methyl alcohol

which the steroid itself could be extracted with little waste. Talc was found to be the best of six diluents tested, and a proportion of 1:100 of steroid to final mixture was chosen. Tests indicate that 95% of the steroid is recoverable by extraction with an appropriate solvent.

The number assigned to each Reference Substance corresponds to a number on a master list of about 400 compounds. The components of set No. 1 was determined largely by (1) the known needs of investigators and (2) availability. Other sets will follow. Suggestions for future additions are welcomed.

Income from the sale of these substances, over and above packaging and distribution costs, will be used for expanding the program, including a possible modest subsidy for preparing additional steroids not otherwise available.

The price is $3.00 for a single steroid and $60.00 for the complete set of 24. All requests for the Steroid Reference Substances should be directed to, and checks made payable to:

U.S.P. Reference Standards,
46 Park Avenue,
New York 16, New York.

STEROID REFERENCE SUBSTANCES

The U.S.P. Board of Trustees has announced a new U.S.P. service made possible through cooperation with the National Institutes of Health of the U. S. Public Health Service. Details of the program are set forth below in the self-explanatory package insert prepared for distribution with the new U.S.P. Steroid Reference Substances to be released shortly. These new substances have been prepared under the direction of the U.S.P. Steroid Advisory Board and will be distributed by authority of the U.S.P. Board of Trustees.

The tremendous advances in the field of endocrinology have focussed attention on the complex steroid substances which are now known to play a vital role in human physiology, both normal and pathologic. The identification of steroid excretion products is highly essential in the diagnosis of certain forms of cancer and furthermore, certain steroids are important therapeutic agents. Many steroids are rare and available from few sources, yet only minute quantities of them are invaluable for purposes of identification in paper chromatography and infra-red spectrophotometry. Thus the Endocrinology Study Section of the National Institutes of Health initiated work which eventually led to the formation of the U.S.P. Steroid Advisory Board and the collection of a first set of U.S.P. Steroid Substances.

The circular provides both general information on the program and specific data on the composition of the 24 steroids comprising the first set. Additional sets will become available as soon as the necessary steroids can be obtained and checked.

It is believed that the availability of these steroids for the purposes stated will be heartily welcomed by endocrinologists both in the clinic and in the laboratory.

In instituting this new service, the U.S.P. Board of Trustees is grateful for the opportunity to make greater use of the well-established distribution facilities by which 52 U.S.P. Reference Standards are provided at cost for pharmaceutical control and research not only within the United States but to all parts of the world.

The Board will appreciate the cooperation of the pharamaceutical and medical press in publicizing this new service.

ADLEY B. NICHOLS, *Secretary*